

**Biophysical studies of protein misfolding and aggregation
in *in vivo* models of Alzheimer's and Parkinson's diseases**

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Abstract

Neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, are characterised by the formation of aberrant assemblies of misfolded proteins. The discovery of disease-modifying drugs for these disorders is challenging, in part because we still have a limited understanding of their molecular origins. In this review, we discuss how biophysical approaches can help explain the formation of the aberrant conformational states of proteins whose neurotoxic effects underlie these diseases. We discuss in particular models based on the transgenic expression of amyloid- β (A β) and tau in Alzheimer's disease, and α -synuclein in Parkinson's disease. Since biophysical methods have enabled an accurate quantification and a detailed understanding of the molecular mechanisms underlying protein misfolding and aggregation *in vitro*, we expect that the further development of these methods to probe directly the corresponding mechanisms *in vivo* will open effective routes for diagnostic and therapeutic interventions.

Keywords: protein misfolding, biophysical methods, Alzheimer's disease, Parkinson's disease, animal models

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1. Introduction

Protein misfolding and aggregation are associated with a wide variety of human diseases (Hardy and Selkoe, 2002; Eisenberg and Jucker, 2012; Jucker and Walker, 2013; Knowles, Vendruscolo and Dobson, 2014; Chiti and Dobson, 2017). These diseases have been linked to a progressive failure of the protein homeostasis system, which controls protein folding, trafficking and degradation (Balch *et al.*, 2008; Kim *et al.*, 2013). The capacity of this system has been shown to decline with age both in model organisms and in humans, which can result in the accumulation of aberrant protein aggregates (Labbadia and Morimoto, 2015). Neurons appear to be particularly vulnerable to such events (Freer *et al.*, 2016; Surmeier, Obeso and Halliday, 2017; Luna *et al.*, 2018; Fu *et al.*, 2019), and the vast majority of neurodegenerative diseases are accompanied by the formation of protein deposits, including those of amyloid- β (A β) and tau in Alzheimer's disease (AD), and of α -synuclein in Parkinson's disease (PD) (Hardy and Selkoe, 2002;

Eisenberg and Jucker, 2012; Jucker and Walker, 2013; Knowles, Vendruscolo and Dobson, 2014; Chiti and Dobson, 2017).

Whereas mature amyloid fibrils are in many cases relatively inert, studies in cell culture and animal models have revealed that misfolded oligomers, which are formed during the process of fibril formation, possess cytotoxic properties (Bucciantini *et al.*, 2002; Baglioni *et al.*, 2006; Haass and Selkoe, 2007; Bemporad and Chiti, 2012; Benilova, Karran and De Strooper, 2012; Chiti and Dobson, 2017). Several mechanisms have been proposed to account for the toxicity of these oligomers, including disruption of lipid membranes, transcriptional deregulation, mitochondrial dysfunction, oxidative stress, proteasome inhibition, and disruption of synaptic plasticity (Haass and Selkoe, 2007; Benilova, Karran and De Strooper, 2012; Roberts and Brown, 2015; De *et al.*, 2019). Some of these mechanisms affect components of the protein homeostasis system, resulting in a collapse of protein quality control and a further increase in protein misfolding and toxicity (Labbadia and Morimoto, 2015). On the whole, however, the molecular mechanisms whereby protein misfolding gives rise to toxicity are not yet understood to the extent needed to enable the development of disease-modifying treatments for the vast majority of these disorders.

In this context, *in vivo* models can both increase our understanding of the mechanisms of disease and provide a platform for drug screening and preclinical development (Dawson, Golde and Lagier-Tourenne, 2018; Götz, Bodea and Goedert, 2018). A variety of model organisms has been exploited to probe protein misfolding diseases, ranging from simple unicellular systems such as yeast, to more complex ones such as rodents and primates. Simple organisms lack specific aspects of human biology, but can be faster and more cost-effective to work with, making them particularly suitable for high-throughput screens, and for the study of highly conserved cellular pathways (Khurana and Lindquist, 2010; Rincon-Limas, Jensen and Fernandez-Funez, 2012; Sin, Michels and Nollen, 2014). More complex organisms can recapitulate human disease phenotypes better, and are typically used in preclinical studies to examine efficacy and safety prior to testing in humans (Jucker, 2010; Sasaguri *et al.*, 2017).

In general, *in vivo* models of disease can be studied at different levels. Organismal and cellular phenotypes can be related to the aggregation states of disease-associated proteins at the molecular level by biochemical and biophysical tools (**Table 1**). Correspondingly, at the systems level, disrupted molecular pathways can be comprehensively monitored by genomics, transcriptomics and proteomics techniques [Barabási, Albert-László, Natali Gulbahce, and Joseph Loscalzo. "Network medicine: a network-based approach to human disease." *Nature reviews genetics* 12.1 (2011): 56-68]. With these approaches, the association of a perturbation (e.g. the expression of a mutant gene) with a phenotype (i.e. an aspect of the disease) is

translated into a knowledge of specific biochemical processes associated with the disease under investigation. Finally, comparison with *in vitro* studies can contribute to deciphering the molecular mechanisms of these processes, and represents a powerful strategy for revealing the molecular origins of a disease, and for identifying possible targets for therapeutic interventions (Knowles, Vendruscolo and Dobson, 2014; Chiti and Dobson, 2017).

Here, we provide an overview of the analysis of protein misfolding in transgenic models of AD and PD based on the transgenic expression of amyloid- β (A β) or tau, and of α -synuclein, respectively. We furthermore discuss methodological challenges in developing biophysical tools to probe *in vivo* the aggregation process and the resulting aggregate species, which are required to provide quantitative assessments of cause-effect relationships in disease and of the efficacy of potential therapies.

2. Determination of protein aggregation mechanisms using *in vitro* biophysical methods

Biophysical studies of protein aggregation *in vitro* enable the identification of possible molecular mechanisms of protein aggregation *in vivo* and the corresponding neurodegenerative processes (Knowles, Vendruscolo and Dobson, 2014; Chiti and Dobson, 2017). This approach is powerful not only because *in vitro* systems are amenable to a wider range of technologies compared to *in vivo* systems (**Table 1**) but also because experimental conditions can be carefully controlled, and the effects of specific perturbations can be examined, e.g. with the introduction of cellular components whose relevance has been revealed by *in vivo* studies.

In particular, the kinetics of the protein aggregation process can be measured quantitatively *in vitro*, leading to detailed mechanistic understanding of the process itself. Protein aggregation over time is typically monitored by measuring the fluorescence of the amyloid-sensitive dye thioflavin T (ThT). The analysis of the concentration dependence of the time courses of the ThT signal leads to a description of the microscopic steps in the aggregation process and the associated rate constants (**Figure 1A**) (Knowles *et al.*, 2009; Cohen *et al.*, 2012; Meisl, Kirkegaard, *et al.*, 2016; Michaels *et al.*, 2017). Aggregation reactions that start from purely monomeric species require primary nucleation events, which involve the assembly of some monomers into disordered oligomers, which can then undergo a structural conversion into forms that have the capacity to grow by elongation through the addition of more monomers [Michaels, T.C.T., Šarić, A., Curk, S. *et al.* Dynamics of oligomer populations formed during the aggregation of Alzheimer's A β 42 peptide. *Nat. Chem.* (2020). <https://doi.org/10.1038/s41557-020-0452-1>]. When fibrils are formed,

secondary processes play a significant role in a variety of systems, and in some cases can dominate the overall aggregation behaviour (S. I. A. Cohen *et al.*, 2013) (Meisl *et al.*, 2014). These processes are dependent on the concentration of the existing amyloid aggregates, and include fibril fragmentation as well as secondary nucleation of monomers on fibril surfaces (**Figure 1B**). Secondary processes represent a feed-forward loop and cause an exponential proliferation of fibrils (S. I. A. Cohen *et al.*, 2013) (Meisl *et al.*, 2014). It is of great importance to understand the relative contributions of the microscopic steps underlying the aggregation process in the context of disease, especially given that potentially toxic oligomers may arise from both primary and secondary nucleation.

We present here recent insights obtained from *in vitro* experiments on the aggregation mechanisms of A β , tau and α -synuclein, in particular those that can be correlated directly with observations made in *in vivo* models.

2.1 A β aggregation

The aggregation of A β into extracellular amyloid plaques is a hallmark of both the familial (early-onset) and sporadic (late-onset) forms of AD. The involvement of amyloid precursor protein (APP) and its sequential processing into A β by β -secretase and γ -secretase (**Figure 2A**) is well established through the amyloid hypothesis (Selkoe and Hardy, 2016). In particular, mutations in APP or in the presenilin (PSEN) subunit of γ -secretase are directly linked to familial forms of the disease, and triplication of chromosome 21, on which the *APP* gene is located, is associated with the development of AD in patients with Down syndrome (Karran and De Strooper, 2016; Selkoe and Hardy, 2016). The relationship between A β aggregation and the onset and progression of AD has been debated extensively (Herrup, 2015; Karran and De Strooper, 2016; Selkoe and Hardy, 2016), with studies suggesting that amyloid deposition may precede the onset of symptoms (Bateman *et al.*, 2012; Jack *et al.*, 2013; Villemagne *et al.*, 2013), and supporting a relationship between A β oligomers and cellular toxicity (Benilova, Karran and De Strooper, 2012; Chiti and Dobson, 2017).

Several A β variants of different lengths and with different post-translational modifications exist in the human brain, with the two most common forms being of 40 (A β 40) and 42 (A β 42) residues (Kummer and Heneka, 2014). Studying *in vitro* the aggregation of these different forms of A β , in particular with respect to oligomer formation, has led to the establishment of correlations between aggregation mechanisms and cytotoxic processes, in particular by revealing the importance of secondary nucleation in the proliferation of the aggregates (S. I. A. Cohen *et al.*, 2013) (Meisl *et al.*, 2014) (**Figure 1A,B**). A β 42 aggregates faster

than A β 40 *in vitro*, with the relative contributions of the microscopic aggregation steps being different for the two isoforms, as in particular the rate of primary nucleation is enhanced for A β 42 (Meisl *et al.*, 2014). From *in vitro* studies, it has furthermore become clear that the two forms can affect the behaviour of each other, although the details of such interplay still remain unclear (Pauwels *et al.*, 2012; Yu-Jen and Yun-Ru, 2014; Cukalevski *et al.*, 2015; Tran *et al.*, 2017).

Familial mutations linked to early-onset AD include those in the presenilins that affect the cleavage of A β , leading to increased levels of A β 42 in particular. However, some mutations also occur within the A β sequence itself, and these have been found to cause increased aggregation propensities (see www.alzforum.org/mutations). A study expressing a series of A β 42 mutants in *Drosophila* has found a remarkable correlation between the *in vitro* aggregation rates of the variants and measures of cytotoxicity, namely reduced locomotor function and lifespan, as described below in Section 3.1 (Luheshi *et al.*, 2007). Using *in vitro* assays, further mechanistic insights have been obtained to probe which of the microscopic steps in the aggregation process are affected for the A β 42 variants. For example, for the A2V A β 42 mutant associated with early-onset AD, it has been shown that secondary nucleation is significantly enhanced compared to that observed for the wild-type form, potentially generating a larger flux of toxic oligomers (Meisl, Yang, *et al.*, 2016). In addition, mutations of residues E22 and D23, which are surface-exposed, have been found to lead to enhanced secondary nucleation, whereas the elongation rates are close to that of the wild-type peptide (Yang *et al.*, 2018). Although the aggregation process may be affected by extrinsic factors *in vivo*, these findings suggest that the surface properties of A β fibrils play an important role in the generation of toxicity.

Given the cytotoxicity of the oligomeric forms of A β (Bucciantini *et al.*, 2002; Baglioni *et al.*, 2006; Haass and Selkoe, 2007; Bemporad and Chiti, 2012; Benilova, Karran and De Strooper, 2012; Chiti and Dobson, 2017), the structural features of these assemblies are under close investigation. Characterisation of a particular form of such oligomers by solid-state NMR spectroscopy (**Table 1**) has provided evidence for parallel β -sheet content (Parthasarathy *et al.*, 2015). Other structural studies on samples prepared *in vitro* have provided evidence for the existence of more loosely packed A β 42 oligomers (Ahmed *et al.*, 2010), as well as antiparallel β -sheet oligomers (Cerf *et al.*, 2009; Stroud *et al.*, 2012; Huang *et al.*, 2015). A structural study of A β 40 oligomers stabilised by zinc ions has also revealed a compresence of both parallel and antiparallel β -sheet conformations (Mannini *et al.*, 2018). Mature A β 42 fibrils, on the other hand, have only ever been found to adopt parallel β -sheet conformations as evidenced by recent structure determination using solid-state NMR spectroscopy (Xiao *et al.*, 2015; Colvin *et al.*, 2016; Wälti *et al.*, 2016) and cryo-

electron microscopy (cryo-EM, **Table 1**) (Gremer *et al.*, 2017). A conformational mapping of A β 42 oligomers using a panel of single-domain antibodies rationally designed to scan the whole A β 42 sequence (Aprile *et al.*, 2017) has revealed that early oligomers produced during the aggregation reaction are structurally different from those produced at later stages, and correspondingly are toxic through different mechanisms (De *et al.*, 2019).

Lipid membranes are likely to play a central role in the aggregation process of A β *in vivo*, given that the peptide is generated from the cleavage of APP within lipid bilayers. As such, aggregation is likely to occur in the vicinity of these membranes, whether intracellularly within endosomes or lysosomes, or extracellularly near the plasma membrane. Depending on the types of lipids and the fluidity of the membranes, studies of synthetic liposomes in *in vitro* aggregation reactions of A β have led to a wide range of different results. Lipids in a gel phase have been found to have a stronger inhibitory effect on A β aggregation kinetics than those in liquid-crystalline bilayers (Hellstrand, Sparr and Linse, 2010). Furthermore, a relationship may exist between the levels of cholesterol and A β deposition, as the major risk factor for late-onset AD, the presence of a mutation in the *ApoE* gene, is involved in cholesterol metabolism (Mahley, 1988). Incorporating cholesterol in lipid vesicles in aggregation assays has revealed that cholesterol does indeed promote A β 42 aggregation (Habchi *et al.*, 2018). This effect could be due to the fact that cholesterol modifies the fluidity of the membrane, although a direct molecular interaction between cholesterol and A β 42 has also been reported (Di Scala *et al.*, 2014). Studying the effects of the lipid membrane composition and fluidity in more detail will be important as these properties may be altered during ageing and neurodegeneration, e.g. as a consequence of lipid oxidation, which not only modifies the lipid types but also reduces membrane fluidity (Borst *et al.*, 2000).

2.2 Tau aggregation

Intracellular neurofibrillary tangles composed of hyperphosphorylated tau are a hallmark of AD, along with the presence of extracellular amyloid plaques. Tau deposits are observed in a family of neurodegenerative disorders collectively known as tauopathies, which in addition to AD include Pick's disease, corticobasal degeneration, progressive supranuclear palsy, argyrophilic grain disease and frontotemporal dementia (Lee, Goedert and Trojanowski, 2001). In the human brain, the *MAPT* gene encoding tau undergoes alternative splicing leading to six possible isoforms, depending on the number of N-terminal insertions (0N, 1N, or 2N) and microtubule binding repeats (3R or 4R) (**Figure 2B**) (Goedert *et al.*, 1989). Mutations in *MAPT* are associated with the development of several tauopathies, although specific mutations have not been reported for AD patients.

Tau was originally described as a protein that binds and stabilises microtubules (Weingarten *et al.*, 1975). It is intrinsically disordered in its monomeric form, and has several phosphorylation sites which can be modified to regulate microtubule stability, with hyperphosphorylation being associated with disease (Wang and Mandelkow, 2016). The classical histopathological staging of the disease (Braak and Braak, 1991), as well as recent advances in positron emission tomography (PET) imaging have revealed that tau deposition is more closely related to the symptoms of AD than is the presence of amyloid plaques (Ossenkoppele *et al.*, 2016; Tosun *et al.*, 2017; Xia *et al.*, 2017). However, the mechanisms by which tau causes cellular toxicity are not fully understood, and the relationship with A β accumulation is still under debate (Spiers-Jones and Hyman, 2014).

The C-terminal region of tau contains the microtubule binding repeats, which include the short hexapeptide stretches VQIINK and VQIVYK that have a high propensity to form β -strands and are essential for the aggregation of tau (Wang and Mandelkow, 2016). Tau is highly hydrophilic and intrinsically disordered, although it has been shown to adopt preferentially a compact conformation in which the N- and C-terminal regions are in close proximity (Jeganathan *et al.*, 2006). The N-terminal part of tau is known as the projection domain, extending away from the interaction site when tau is bound to microtubules, and away from the fibril surface in the case of fibrillar tau. Given that truncated versions of tau, or constructs comprising only the C-terminal repeat region, have increased aggregation propensity, the N-terminal region is thought to exert a protective effect (Wang and Mandelkow, 2016). Under typical *in vitro* conditions, however, tau does not aggregate spontaneously, but to form fibrils requires the addition of catalysts, as for example poly-anionic molecules such as heparin. The negative charges may be required to overcome the repulsion between tau molecules, each of which carries a net positive charge in the C-terminal repeat region, although heparin also induces the aggregation of phosphorylated tau with a net negative charge by an unknown mechanism (Wang and Mandelkow, 2016).

In vitro studies have been instrumental in complementing *in vivo* work to shed light on the role of familial mutations, splicing and phosphorylation in tau aggregation and toxicity. A key finding has been that disease-associated mutations reduce the affinity of tau for microtubules, which may increase the risk of aggregation *in vivo* as the same region of the protein is involved in both processes (Hong *et al.*, 1998). The six tau isoforms have been shown to vary in their aggregation propensity, with the 4R isoforms being more aggregation-prone than the 3R isoforms (Zhong *et al.*, 2012). How this finding relates to pathological conditions is unclear, however, as in AD a mixture of 3R and 4R isoforms is found in tau tangles, whereas in other tauopathies such as Pick's disease and agyrophilic grain disease either the 3R or 4R tau species

dominates. A potential explanation could again be provided by post-translational modifications such as phosphorylation. Out of the 85 theoretical phosphorylation sites in the tau sequence, about 45 have been observed in tau derived from AD brains (Hanger, Anderton and Noble, 2009). *In vitro* assays have led to the identification of the potential kinases and phosphatases associated with various sites, and for example GSK-3 has been found to be capable of targeting many sites (Hanger, Anderton and Noble, 2009), consistent with its important role in tau toxicity as observed in a variety of animal models as outlined in Section 3.2. In the light of the complex patterns of phosphorylation, it is not surprising that conflicting results have been obtained on whether hyperphosphorylation of tau promotes or inhibits its aggregation. Hyperphosphorylated tau extracted from AD brains is able to form fibrils *in vitro*, whereas dephosphorylation using alkaline phosphatase inhibits aggregation into fibrils (Alonso *et al.*, 2001). Conversely, pseudo-phosphorylated constructs comprising the tau repeat region show much slower aggregation *in vitro*, although they may result in the formation of a larger number of oligomers (Kumar *et al.*, 2014).

Directly and quantitatively observing the formation of oligomeric species has been challenging due to their heterogeneous and transient nature, but an elegant single-molecule Förster resonance energy transfer (smFRET, **Table 1**) assay has yielded important insights into the generation of tau oligomers (Shammas *et al.*, 2015). In this assay, relatively small oligomers were detected early in the aggregation process and these were found to subsequently convert into fibrils. The familial mutants Δ K280 and P301L were not only observed to aggregate faster, but also to form a larger population of oligomers than the wild-type protein, the oligomers being of the same apparent size. An anti-aggregation mutant with two proline substitutions that is less toxic in mouse models (Eckermann *et al.*, 2007; Mocanu *et al.*, 2008) was also found to form oligomers, however. Thus, the levels of oligomeric species cannot be directly correlated with toxicity, presumably because the mutations lead to oligomers with distinct structural characteristics (Shammas *et al.*, 2015). In a follow-up study, different types of oligomers could be distinguished by varying the buffer conditions in the smFRET system (Kjaergaard *et al.*, 2018). One type was found to be sensitive to the ionic strength of the buffer, and to be in rapid exchange with tau monomers, whereas the other type was found to be more stable and presumably unable to convert to fibrils (Kjaergaard *et al.*, 2018). Both species may be toxic, but it is likely that they operate via different mechanisms.

Recently, liquid-liquid phase separation has attracted attention as a biophysical phenomenon that may act as a precursor to the aggregation of several disease-related proteins (Shin and Brangwynne, 2017; Alberti and Dormann, 2019). Also the repeat region of tau has been found to condense into liquid droplets *in vitro*, depending on parameters such as the protein concentration, pH value and temperature (Ambadipudi *et al.*,

2017). Phase separation has been observed to induce reversibly the formation of β -strands as detected by circular dichroism (CD, **Table 1**) spectroscopy. The absence of ThT fluorescence suggests that the phase-separated state does not consist of amyloid fibrils, however, although the addition of heparin to the droplets was found to induce amyloid-like aggregation. NMR spectroscopy (**Table 1**) has furthermore shown that the conformation of tau is not drastically altered in the phase-separated state, whereas paramagnetic relaxation experiments and ^{13}C -detected experiments have provided evidence for involvement of the repeat regions and the hexapeptide motifs (Ambadipudi *et al.*, 2017, 2019).

Full-length, phosphorylated tau has also been found to phase separate under physiological conditions and to form droplets both *in vitro* and in neuronal cells in culture (Wegmann *et al.*, 2018). The process has been suggested to be mediated by weak hydrophobic interactions between the hexapeptide repeats, as the liquid droplets were observed to be sensitive to dissociation by urea and by 1,6-hexanediol. The N-terminal region of tau by itself was also observed to phase separate, but in this case driven by electrostatic interactions that can be disrupted by salt. The importance of electrostatics is further underscored by the effect of tau phosphorylation, which was shown to be required for efficient droplet formation. Finally, tau liquid droplets were found to convert rapidly into gels, and after prolonged incubation into non-spherical ThS positive aggregates, confirming that tau phase separation can be on pathway to aggregate formation (Wegmann *et al.*, 2018). Whether phase separation drives tau aggregation in human disease remains to be determined, and it will be exciting to study this phenomenon in *in vivo* models, e.g. by using fluorescence lifetime imaging microscopy (FLIM, **Table 1**) in *C. elegans* (Laine *et al.*, 2019) (see Section 3.4).

2.3 α -Synuclein aggregation

The 140-residue protein α -synuclein (**Figure 2C**) is the major component of the insoluble intracellular deposits known as Lewy bodies and Lewy neurites that are characteristically found in the brains of PD patients, as well as in those suffering from dementia with Lewy bodies (DLB) (Spillantini, R. A. Crowther, Jakes, Hasegawa, *et al.*, 1998) and multiple systems atrophy (MSA) (Spillantini, R. A. Crowther, Jakes, Cairns, *et al.*, 1998), a set of diseases collectively termed synucleinopathies. PD is associated with the specific loss of dopaminergic neurons in the substantia nigra, which results in impaired motor function, as well as a variety of other deficits including behavioural changes, sleep problems, and a diminished sense of smell. Gene duplications and triplications and several point mutations in the *SNCA* gene, encoding α -synuclein, in hereditary forms of PD have supported a causative role for its aggregation in the disease process (Kalineri, Bostantjopoulou and Fidani, 2016), although the molecular mechanisms by which this pathology occurs are still poorly understood.

Many of the biophysical properties of α -synuclein that have been established through *in vitro* studies have been shown to be valuable in interpreting *in vivo* models. *In vitro* aggregation assays using recombinant α -synuclein have shown that its spontaneous aggregation is negligible at neutral pH, although when preformed seeds are added the reaction can proceed rapidly (Buell *et al.*, 2014). At slightly acidic pH, secondary processes in which existing fibrils catalyse the formation of new ones on their surfaces start to dominate (Buell *et al.*, 2014). Such conditions may be relevant in cellular compartments such as lysosomes and synaptic vesicles. Furthermore, the presence of small unilamellar vesicles (SUVs) in the aggregation assay dramatically increases primary nucleation as α -synuclein binds to the lipid bilayers, which initiates the aggregation process (**Figure 1C,D**) (Galvagnion *et al.*, 2015, 2016). In agreement with the propensity of α -synuclein to interact with lipid membranes, genetic screens have systematically identified proteins involved in vesicle trafficking to be related to α -synuclein-mediated toxicity, as described in Section 4.2. These findings do not seem to arise simply as a result of the expression of α -synuclein in non-neuronal cell types such as yeast or *C. elegans* body wall muscle cells, as many of the proteins identified in the screens have been confirmed in neuronal models and in neurons from higher organisms (Cooper *et al.*, 2006; Gitler *et al.*, 2008; Hamamichi *et al.*, 2008).

Although the presence of mature Lewy body pathology does not appear to be essential for toxicity to be observed in the vast majority of *in vivo* models as discussed in Section 3.4, the process of protein aggregation is likely to play a causative role in synucleinopathies, given that the more aggregation prone α -synuclein variants such as A53T (Conway *et al.*, 2000) or the C-terminal truncation fragment (Crowther *et al.*, 1998) are typically associated with increased toxicity. Data on the A30P mutant are, however, more difficult to interpret, as its aggregation kinetics measured *in vitro* are very similar to those of the wild-type protein (Flagmeier *et al.*, 2016), yet it does not readily form inclusions in yeast (Outeiro and Lindquist, 2003) or in *C. elegans* body wall muscle cells (Perni *et al.*, submitted), although A30P aggregates were demonstrated to form in fly (Feany and Bender, 2000) and rodent (Klein *et al.*, 2002; Lo Bianco *et al.*, 2002; Lauwers *et al.*, 2007; da Silveira *et al.*, 2009) models. These differences may be related to the reduced affinity for lipid bilayers of this latter variant (Bodner *et al.*, 2010). It will be interesting to study these mechanisms in more detail and also to carry out studies *in vivo* of the more recently identified familial PD mutants E46K, H50Q and G51D, which appear to be less aggregation prone *in vitro* (Flagmeier *et al.*, 2016), to shed more light on the aggregation process with respect to toxicity.

Oligomers have been suggested to play a key role in the toxicity of α -synuclein, although their molecular features *in vivo* have remained elusive. Stable α -synuclein oligomers can be generated *in vitro* and are toxic to neuronal cells in culture, causing increased levels of reactive oxygen species (Cremades *et al.*, 2012; Chen *et al.*, 2015). Detailed structural examination of stable toxic compared to non-toxic α -synuclein oligomers has revealed that toxicity is linked to strong binding to membrane surfaces and to insertion of a well-structured core into the lipid bilayer, whereas the more disordered species do not enter the bilayer and are not toxic (Fusco *et al.*, 2017). This proposed mechanism of toxicity is interesting in the context of the interference with vesicle trafficking and lipid metabolism that was found in the *in vivo* models of α -synuclein as described below in Section 4.2, highlighting the power of connecting the observations made in cellular and animal models with detailed biophysical characterisation *in vitro* to understand the mechanisms behind protein misfolding diseases.

3. *In vivo* models of protein misfolding diseases

A common way to create an *in vivo* model of a protein misfolding disease is through the transgenic expression of a human gene associated with the disease in a non-human organism. The gene may typically encode for the protein that misfolds and aggregates, or for a protein that regulates this process. These transgenic models are often based on mutant genes known from familial cases of the disease in order to obtain a strong phenotype with high penetrance. Overexpression is usually employed to obtain high levels of the disease-associated protein, which facilitates the detection of a response in the organism.

Commonly used methods of probing the process of protein misfolding and aggregation in transgenic models include staining with amyloid-binding dyes or conformation-specific antibodies, ultrastructural examination by electron microscopy (EM), and biochemical assays based on the solubility, the size or the shape of the misfolded species (**Table 1**). In parallel, the phenotype of the model can be assessed both at the organismal and the cellular levels, with the goal of correlating the conformational state of the disease-associated protein to neurotoxicity. In this section, we discuss this approach in the context of yeast, worm, fly and rodent models based on the transgenic expression of A β , tau, or α -synuclein (**Figure 3**).

3.1 A β models

Yeast models

Yeast, in particular *Saccharomyces cerevisiae*, is one of the simplest and most-studied eukaryotic organisms available for the study of cellular pathologies (Verduyck *et al.*, 2016; Tenreiro *et al.*, 2017; Seynnaeve *et al.*, 2018; Rencus-Lazar *et al.*, 2019). This organism has a short generation time, and an arsenal of research tools is available for its study (Smith and Snyder, 2006). *S. cerevisiae* was the first eukaryote to have its complete genome sequenced (Goffeau *et al.*, 1996), and we now know that at least 60% of the *S. cerevisiae* genes have significant homology, or share conserved domains, with human genes (Khurana and Lindquist, 2010). However, since not all homologues have conserved functions, and not all human disease-related proteins have a yeast homologue, an often-used strategy to create a disease model is to express the human disease-related gene in yeast.

Yeast lacks known homologues of APP and β - and γ -secretases, yet transgenic variants of this organism have been successfully employed to investigate APP processing (Zhang *et al.*, 1994, 1997; Lüthi *et al.*, 2003) and to screen for inhibitors of β -secretase (Middendorp *et al.*, 2004) and A β -mediated toxicity (Bharadwaj *et al.*, 2012; Amen and Kaganovich, 2016; Seynnaeve *et al.*, 2018). It has proven challenging, however, to generate yeast models to observe the full A β aggregation process and its associated toxicity (Verduyck *et al.*, 2016; Seynnaeve *et al.*, 2018). Expression of A β 42 in fusion with green fluorescent protein (GFP-A β 42) in this organism was found to result in small punctate patches in newly transformed cells, which become larger as the cells age, and a modest reduction of about 5% in cellular growth was observed (Caine *et al.*, 2007). Cells expressing an A β 42 fusion protein with the MRF domain of the yeast prion Sup35p have been reported as a model system for A β 42 oligomerization, where a decrease in activity of the reporter was shown to be associated with the formation of sodium dodecyl sulfate (SDS)-stable oligomers (Bagriantsev and Liebman, 2006).

To model the generation of A β in the secretory pathway, experiments have been carried out using the peptide fused to an ER-targeting sequence (Treusch *et al.*, 2011). This model was shown to lead to the accumulation of A β in the secretory pathway, accompanied by growth defects, which were more severe for A β 42 than for A β 40. Consistent with these observations, A β 42 was shown to form more oligomers than A β 40. These observations were confirmed in an independent study in which the expression of A β 42 with the ER-targeting sequence was furthermore shown to lead to impaired mitochondrial function and proteasomal activity (Chen and Petranovic, 2015). Further to these results, expression of an MF α -A β 42-GFP fusion construct that also enters the secretory pathway and causes toxicity was found to be exacerbated for the familial, more aggregation-prone E22G Arctic mutant (D'Angelo *et al.*, 2013), although the

aggregation state of the fusion proteins was not examined. An overview of the various yeast models used in the study of A β aggregation can be found in **Table 2**.

Worm models

The nematode worm *C. elegans* has been used extensively to investigate the molecular processes underlying the cytotoxicity of A β aggregation (Link, 2006; Alexander, Marfil and Li, 2014), and for the discovery of compounds capable of inhibiting such processes (Habchi *et al.*, 2016; Perni *et al.*, 2018). *C. elegans* has the advantage over yeast that it is multicellular, yet has a relatively simple body plan with 959 cells for the adult hermaphrodite, including a nervous system comprised of 302 neurons. The complete genome of *C. elegans* was sequenced in 1998 (The *C. elegans* sequencing consortium, 1998), and follow-up studies showed that 38% of the *C. elegans* genes have known human homologues (Shaye and Greenwald, 2011). The nematode typically lives up to 2-3 weeks in laboratory conditions, a convenient experimental timescale, and it has been shown to exhibit age-associated phenotypes, such as deterioration of muscle structure, as observed in higher organisms including humans (Herndon *et al.*, 2002).

Although *C. elegans* contains the *APP* homologue *apl-1*, this gene lacks a sequence region homologous to human A β . Moreover, since *C. elegans* lacks the β -secretase activity required to process full-length APP, transgenic models have been largely based on the overexpression of the human A β 42 sequence (**Table 3**). In the first reported model, A β 42 was placed under control of the *unc-54* promoter for expression in the body wall muscle cells (Link, 1995). For efficient expression, a signal sequence upstream of A β was found to be required, and the peptide was shown to be properly processed, yet A β deposits were found in the cytoplasm of the muscle cells rather than in the secretory system or in the extracellular space (Link *et al.*, 2001). However, co-immunoprecipitation has revealed that A β interacts with the ER-homologue of the Hsp70 chaperone in this model, suggesting that it enters the secretory pathway, but is then rerouted to the cytoplasm for degradation (Fonte *et al.*, 2002). Examination using amyloid-specific dyes and immunoelectron microscopy (EM) has shown that a subset of the deposits is fibrillar, whereas a dimeric A β construct forms deposits but without fibrillar structures (Fay *et al.*, 1998; Link *et al.*, 2001). From a set of mutants designed to interfere with β -strand formation, the L17P and M35C variants also fail to produce deposits that stain with amyloid-sensitive dye thioflavin S (ThS) (Fay *et al.*, 1998).

Subsequently, it was found that in the original model A β is processed to residues 3-42 (McColl *et al.*, 2009), a fragment that is a major component of human amyloid plaques with the N-terminal residue modified to a pyroglutamate (Harigaya *et al.*, 2000). A model has therefore been created with two extra residues in

between the signal sequence and that of A β , resulting in expression of the 1-42 peptide (McColl *et al.*, 2012). In both strains, amyloidogenic protein aggregation (**Figure 3D**, left panel) results in paralysis of the worms within several days of adulthood, yet the strain expressing A β (1-42) is more temperature sensitive and shows increased paralysis at higher temperatures, perhaps reflecting differences in the aggregation behaviour of the two isoforms (McColl *et al.*, 2012).

A model in which A β (3-42) is expressed in neurons features oligomeric species recognised by the NU-4 antibody, and displays a phenotype with impaired chemotaxis (Wu *et al.*, 2006) and defects in associative learning (Dosanjh *et al.*, 2010). Interestingly, these worms are defective in sensing benzaldehyde, but not diacetyl, suggesting that the vulnerability of the associated sensory pathways towards A β toxicity differs. In a more recent model expressing the corrected A β (1-42) variant throughout the nervous system, the protein was found to accumulate in the insoluble fraction during ageing, whereas neuromuscular defects and behavioural abnormalities were observed earlier on (Fong *et al.*, 2016). Expressing A β specifically in glutamatergic neurons results in visible neurodegeneration of the glutamatergic neurons in the tail of the worm (Treusch *et al.*, 2011). However, a model designed in our own laboratory that selectively expresses low levels of A β 42 in the two BAG sensory neurons, which are also glutamatergic, shows moderate behavioural defects in a CO₂ sensing assay without overt signs of neurodegeneration, perhaps because these neurons are more resistant to toxic protein aggregation than others (Sinnige *et al.*, 2019). Notably, in this model A β 42 appears to be localised mainly in the cell body (**Figure 3D**, right panel) and does not visibly form aggregates, supporting this notion.

Fly models

D. melanogaster, the fruit fly, is a common model organism for neurodegenerative diseases (Rincon-Limas, Jensen and Fernandez-Funez, 2012; Prüßing, Voigt and Schulz, 2013) (**Table 4**), whose main advantage over *C. elegans* is its more sophisticated nervous system. The central nervous system of the fly comprises glia as well as neurons, and contains specialised sub-structures such as a compound eye and mushroom bodies for olfactory learning and memory, both of which are often employed in disease models. Limitations for the modelling of neurodegenerative disorders, on the other hand, are the absence of an adaptive immune system, and a lack of blood vessels.

Like *C. elegans*, *Drosophila* lacks a sequence region corresponding to human A β in its APP homologue. A β -secretase homologue does exist (Carmine-Simmen *et al.*, 2009), but has low levels of activity (Fossgreen *et al.*, 1998; Greeve *et al.*, 2004). γ -secretase activity has also been demonstrated by expression in

Drosophila of a construct comprising the A β region, the transmembrane region and the C-terminal segment of human APP, leading to generation of a 4 kDa A β fragment that may correspond to either A β 40 or A β 42 (Fossgreen *et al.*, 1998). A transgenic model for A β overproduction has been designed in which human APP is expressed together with human β -secretase in the eye, resulting in the generation of a 4 kDa A β peptide and progressive degeneration of the retina and neuropil regions (Greeve *et al.*, 2004). ThS-positive inclusions with a star-like appearance have been found in older flies, yet neurodegeneration precedes the formation of these amyloid deposits. Co-expression of human APP and β -secretase with *Drosophila* presenilin containing mutations corresponding to those in familial AD was found to reduce the onset of amyloid deposition remarkably, from 68 days to 37 days, and to cause more severe neurodegeneration (Greeve *et al.*, 2004).

Expression of human APP and β -secretase throughout the *Drosophila* nervous system was found to result in A β deposition in the cortical layer, as shown by antibody staining, and memory defects in young flies, followed by extensive neurodegeneration in older flies (Sarantseva *et al.*, 2009). Using a similar approach, a model has been generated that displays a more severe phenotype, including abnormal wing morphology at the time of eclosion and strong neuroanatomical changes, suggesting a developmental defect. In this model, punctate A β deposits, which stain with the amyloid-binding dye X-34, are present in relatively young flies (Chakraborty *et al.*, 2011). The reason for the discrepancy between these two models has been suggested to lie in the different caloric intake of the flies, which may affect γ -secretase processing (Chakraborty *et al.*, 2011). Raising the flies at lower temperature was found to result in a milder phenotype, with A β deposits accumulating during ageing, accompanied by impaired climbing and memory functions and visible neurodegeneration (Mhatre *et al.*, 2014).

To avoid the complications associated with APP processing, another approach has been exploited in which A β 40 and A β 42 are directly expressed following the pre-proenkephalin signal peptide, which was shown to be cleaved correctly (Finelli *et al.*, 2004). Flies expressing A β 42 have a rough eye phenotype and the peptide accumulates in the insoluble protein fraction, whereas flies expressing the less aggregation-prone A β 40 appear normal (Finelli *et al.*, 2004). The lines that were created have varying expression levels of A β 42 in the eye, depending on the integration site of the construct and the number of copies. Aggregation and toxicity were found to be proportional to the expression levels, with low A β 42 levels showing small deposits and modest neurodegeneration, and higher levels resulting in larger deposits and more severe morphological defects (Finelli *et al.*, 2004).

Expression of A β 42 throughout the nervous system leads to locomotory and other behavioural deficits in relatively young flies, in addition to a dramatically reduced lifespan (Finelli *et al.*, 2004). Further characterisation of this model has revealed that A β 42 forms deposits in various regions of the brain, a subset in the region of the Kenyon cell bodies being ThS positive (**Figure 3F**) (Iijima *et al.*, 2004). However, fibrils could not be identified by EM, suggesting that A β 42 forms diffuse, relatively unstructured deposits rather than mature amyloid plaques in this model. Similar experiments with A β 40 have shown that it accumulates in the same brain areas, but deposits are not visible. Whereas both A β 40 and A β 42 expression cause age-associated learning defects, only A β 42 results in locomotory defects and neurodegeneration at later ages. This observation suggests that visible protein aggregation is required in this model to observe neurodegeneration, but not neuronal dysfunction, which could perhaps be mediated by oligomers (Iijima *et al.*, 2004).

Using the signal peptide of the *Drosophila necrotic* gene, lines have been generated expressing A β 40 and A β 42, and also A β 42 with the Arctic mutation E22G in the *Drosophila* nervous system (Crowther *et al.*, 2005). All A β variants were found initially to accumulate in intracellular deposits, which react with an antibody specific to oligomeric A β (Crowther *et al.*, 2005). In older flies, A β 42 shows extracellular deposits accompanied by vacuolar structures, which are exacerbated in the E22G mutant, whereas they do not occur in A β 40 flies. The extracellular deposits do not stain with Congo red, indicating that they do not consist of mature amyloid fibrils, but might resemble diffuse plaques as in the previously described fly model (Iijima *et al.*, 2004). The A β 42 flies have a rough eye phenotype and locomotory defects, which occur before the appearance of the extracellular deposits, and the phenotype is exacerbated by the Arctic mutation. Furthermore, the A β 42 flies have a shorter lifespan than controls, whereas the survival of the A β 40 flies is indistinguishable from controls. The phenotype of the A β 42 flies can be partially restored by treatment with the amyloid-specific dye Congo red, suggesting that toxicity is directly caused by A β 42 aggregation.

In a subsequent study, a panel of A β 42 mutants with different aggregation propensities has been expressed in the *Drosophila* nervous system. The computationally predicted aggregation rates were found to correlate strongly with lifespan and locomotory function, especially when the propensity to form protofibrillar (oligomeric) aggregates was considered (Luheshi *et al.*, 2007). Similarly, whereas expression of A β 40 itself is relatively harmless to the flies, point mutations that increase the formation of prefibrillar A β 40 species *in vitro* result in a toxic phenotype when expressed in *Drosophila* (Brorsson *et al.*, 2010). Furthermore, an engineered affibody has been shown to restore the phenotype of A β 42 flies, by binding to the monomer and targeting it for degradation (Luheshi *et al.*, 2010). On the other hand, the KW1 antibody, which is

specific to A β 40 oligomers, induces aggregation into toxic protofibrillar species, shortening the lifespan of the flies (Wacker *et al.*, 2014). Further evidence for the role of intermediate species in toxicity has been provided by the expression of tandem constructs of A β 40 and A β 42, in which two monomers are connected with a linker to increase the effective concentrations and aggregation rates. Expression of both tandem constructs leads to the formation of deposits in the fly brain, but only the tandem A β 42 flies show a dramatic reduction in lifespan relative to those expressing the monomeric peptide, an effect that is associated with an increase in SDS-soluble species (Speretta *et al.*, 2012).

A β 42, the E22G Arctic mutant, as well as a designed mutant with lower aggregation propensity have furthermore been expressed in the *Drosophila* nervous system using the pre-proenkephalin signal peptide (Iijima *et al.*, 2008). In this study, the A β variants were found to be localised intracellularly, residing in the endoplasmic reticulum (ER), Golgi and lysosomes, but in contrast to the model by Crowther and co-workers, no oligomer-specific antibody staining was observed. None of the lines was found to show Congo red staining, demonstrating that mature fibrils are not noticeably formed either. The Arctic mutant shows an increased population of dimers and trimers by western blot, a larger number of ThS-positive deposits in the cell bodies accompanied by neuronal loss, a shortened lifespan and stronger locomotory defects than the model expressing wild-type A β 42. The designed mutant, however, forms more ThS-positive deposits in the neurites leading to neuropil loss, and has a longer lifespan, yet shows learning defects similar to the Arctic mutant. These discrepancies have been suggested to arise from a differential intracellular distribution of the variants, in addition to their different aggregation propensities, highlighting the importance of the biological environment in mediating cellular toxicity (Iijima *et al.*, 2008).

Further studies on flies expressing A β variants in the giant fiber neurons have demonstrated that intracellular A β , which accumulates in cell bodies and axons, leads to impairments in synaptic function, accompanied by reduced numbers of mitochondria and synaptic vesicles (Zhao *et al.*, 2010). Again, the severity of the phenotype was found to be correlated with expression levels and to be stronger for the Arctic mutant. In addition, it has been shown that levels of the synaptic protein bruchpilot and presynaptic calcium channels decline with age in flies expressing the Arctic mutant, compared to controls (Huang *et al.*, 2013). This *Drosophila* model expressing A β in a subset of neurons has thus proved to be suitable to study perturbations at the single-neuron level, and has provided strong evidence for a connection between A β accumulation and synaptic dysfunction even in this relatively simple model organism.

Rodent models

One of first transgenic mouse models of AD shown to develop amyloid plaques resembling those in the human disease relied on the overexpression of the V717F mutant of human APP, which is associated with early-onset AD, driven by the platelet-derived growth factor β (PDGF- β) promoter (Games *et al.*, 1995). These mice, referred to as PDAPP from the combination of PDGF- β and APP, show ThS-positive extracellular plaques accompanied by dystrophic neurites and reactive astrocytes. The plaques vary in morphology from round to diffuse. The pathology is age-related, with plaques first appearing between 6 and 9 months of age, and increasing in number as the mice age further. This model displays synaptic loss correlated with increased plaque deposition in older mice (Dodart *et al.*, 2000) as well as learning defects (Chen *et al.*, 2000), but without overt neuronal loss (Irizarry *et al.*, 1997).

A wide variety of models based on mutant APP overexpression has been subsequently generated, which similarly display amyloid plaques and are associated with synaptic dysfunction and gliosis (reviewed in (Elder, Gama Sosa and De Gasperi, 2010; Hall and Roberson, 2012; Drummond and Wisniewski, 2017); see www.alzforum.org for a full list of available rodent models and **Table 5** for those discussed here). These models differ from one another in various ways including the ratio of A β 40 to A β 42, the morphology of the plaques, the age at which they appear and the brain regions that are affected. A widely used model is the Tg2576 mouse, in which human APP is overexpressed with the Swedish double mutation K670N/M671L using the prion protein (PrP) promoter that drives strong expression in neurons (Hsiao *et al.*, 1996). In this model, dense ThS-positive deposits are found in older mice (11-13 months), a subset of which display Congo red birefringence. Dystrophic neurites have also been found in this case, as well as reactive astrocytes and microglia. At the age where plaques are apparent, the mice show behavioural impairment in the Morris water maze test. However, there is no direct correlation between the quantity of plaques and cognitive defects, as the behavioural phenotype remains stable between 6 and 14 months, while the plaque load continues to increase. Rather, the levels of a 56 kDa soluble A β species, referred to as A β *56, identified in extracts from Tg2576 mice by SDS PAGE and size exclusion chromatography (SEC) (**Table 1**) correlate with memory impairment (Lesné *et al.*, 2006). Furthermore, this species affects long-term memory when purified and infused into young rats, supporting the hypothesis that A β oligomers are responsible for neurotoxicity in AD.

A complication of APP-based transgenic mouse models is that its processing gives rise to several variants of the A β peptide, as well as other intracellular and extracellular fragments of APP (**Figure 2A**), which may all contribute to the observed phenotype. To address specifically the role of A β accumulation, a different approach has been taken in expressing constructs comprising the BRI protein fused to either A β 40

or to A β 42, which are released by the furin cleavage site directly preceding the A β sequence (McGowan *et al.*, 2005). Expression of the A β 40 construct leads to high levels of free peptide, which however remains soluble, so that no amyloid plaques are observed. In contrast, BRI-A β 42 mice show amyloid pathology starting at 3 months of age, including the presence of diffuse and compact ThS-positive plaques, as well as deposits in blood vessels in the brain, termed cerebral amyloid angiopathy (CAA). However, although dystrophic neurites and reactive astrogliosis have been observed surrounding the plaques, this study reported that the mice have no behavioural abnormalities, suggesting that A β 42 deposition by itself is not sufficient to trigger neurotoxicity, at least up to the age investigated (McGowan *et al.*, 2005). It is interesting to compare these findings to those from the *Drosophila* and *C. elegans* models discussed above, where A β fusions do lead to behavioural phenotypes. These observations can perhaps be attributed to toxicity arising from intracellular rather than extracellular A β in these worm and fly models. Intracellular A β may also be present in APP-based mouse models (LaFerla, Green and Oddo, 2007), but not in those developed using the BRI fusion approach.

Mutations in the presenilin genes *PSEN1* and *PSEN2* associated with early onset forms of AD have been employed to increase the levels of the more aggregation-prone A β 42 in mouse models. Expression of familial mutant, but not wild-type, PSEN1 has been shown to increase specifically the levels of A β 42 over A β 40 in mice (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Citron *et al.*, 1997), and crossing these lines with APP mutant mice has led to accelerated A β deposition (Borchelt *et al.*, 1997; Holcomb *et al.*, 1998). Several PSAPP mouse models based on different combinations of APP and PSEN1/2 mutants exist, and all show similar pathologies to that of the APP transgenic mice, but at an earlier age and accompanied by a stronger behavioural phenotype. They also suffer from greater neuronal loss, although not at levels comparable to the widespread degeneration in human AD (McGowan, Eriksen and Hutton, 2006; Elder, Gama Sosa and De Gasperi, 2010; Hall and Roberson, 2012). For example, the PS2APP mouse model, a cross of the APP K670N/M671L mutant and PSEN2 with the N141I mutation, displays congophilic amyloid plaques at 9 months of age, which are surrounded by dystrophic neurites and activated microglia and astrocytes (Richards *et al.*, 2003). However, deficiencies in synaptic plasticity and behavioural tests such as the Morris water maze are relatively mild (Richards *et al.*, 2003).

In the APP^{SL}PS1KI mouse model, which expresses APP with V717I and K670N/M671L mutations, and PSEN1 with M233T and L235P mutations, large numbers of plaques are already apparent at 6 months of age, and about 50% neuronal loss in the CA1/2 layer of the hippocampus has been observed at 10 months of age (Casas *et al.*, 2004). The loss of neurons appears to correlate better with the presence of intracellular

A β aggregates that can be stained with ThS than with extracellular plaques, which is interesting given the evidence for the presence of intracellular A β in human AD (LaFerla, Green and Oddo, 2007). Coinciding with this pathology, the mice become increasingly impaired in working memory and motor tasks from 6 months of age (Wirths *et al.*, 2008). Apart from the fact that this model produces A β 42 as the dominant A β species, another interesting feature in relation to human AD is the progressive N-terminal truncation and modification of A β 42 into species that have been detected in patient material (Casas *et al.*, 2004).

A model with very extensive amyloid deposition has been created using the L166P mutant form of PSEN1, which has led to a disease onset as young as 24 years of age in humans, crossed with a mouse strain expressing APP K670N/M671L (Radde *et al.*, 2006). In the resulting model, congophilic amyloid plaques appear in the neocortex at 6 weeks, whereas deposits in different regions of the hippocampus occur between 2 and 5 months. As in the other mouse models, the plaques are surrounded by dystrophic neurites and glia, but cognitive impairment is only evident at 8 months (Radde *et al.*, 2006).

The approach of combining APP and PSEN mutants has been further extended through the generation of a mouse strain with a total of five familial AD mutations, the 5xFAD mouse, which has become one of the most frequently used models in AD research (Oakley *et al.*, 2006). Constructs of APP with K670N/M671L, I716V and V717I mutations, and PSEN1 with M146L/L286V, were co-injected to create this model, resulting in rapid accumulation of A β 42 and ThS-positive plaques from 2 months of age. The plaques are smaller but more numerous than in the Tg2576 mouse, consistent with an increase in nucleation events due to the higher concentration of A β 42. In this model, intracellular A β aggregation also appears to play a role, as indicated by small ThS-positive puncta in pyramidal neurons. It has been speculated that extracellular plaques could arise from these aggregates upon cell death, as indeed degenerating neurons with intracellular deposits are sometimes found in the vicinity of plaques. These regions of co-localisation are correlated with synaptic degeneration and significant loss of neurons. At the behavioural level, there is no phenotype at 2 months in these mice, but at 4-5 months, memory defects are apparent in the Y-maze test (Oakley *et al.*, 2006).

Mouse models based on the overexpression of human genes produce artificially high protein levels, including other APP fragments in addition to A β , including those coming from the α -secretase pathway, some of which may also be amyloidogenic [Vandersteen, Annelies, et al. "A comparative analysis of the aggregation behavior of amyloid- β peptide variants." FEBS letters 586.23 (2012): 4088-4093], and altering in complex ways the protein homeostasis system [Saito, Takashi, et al. "Calpain activation in Alzheimer's model mice is an artifact of APP and presenilin overexpression." Journal of Neuroscience 36.38 (2016):

9933-9936], altogether complicating the interpretation of the phenotype. Second generation mouse models remedy some of these issues by making use of knock-in strategies to produce human A β in the context of endogenous mouse APP (Sasaguri *et al.*, 2017). For example, the A β sequence of mouse APP was humanised, and Swedish (K670M/M671L) and Iberian (I716F) mutations were introduced in order to generate high levels of preferentially A β 42 (Saito *et al.*, 2014). The resulting APP^{NL-F} mice display progressive A β pathology and inflammation, accompanied by memory impairment in the Y-maze test. Adding the Arctic mutation E693G has resulted in the APP^{NL-G-F/NL-G-F} model that displays earlier A β deposition and a stronger inflammatory response.

Another strategy to avoid overexpression consists in triggering an amyloid cascade response by intracerebral infusion of A β seed aggregates produced either *in vitro* or derived from cell systems, other animal models, or AD patients (Jucker and Walker, 2018). These non-transgenic mouse models are particularly useful in the study of the mechanisms of propagation of the aggregates across cells and brain tissues, but the infusion process tends to generate inflammatory reactions that complicate the interpretation of the results.

Given that rats are evolutionarily more closely related to humans than mice, and display more complex patterns of behaviour, rat models have certain advantages for recapitulating and testing the pathogenic mechanisms behind AD. Although early rat models transgenic for APP did not display amyloid plaques, probably due to insufficient levels of expression (reviewed in (Carmo and Cuello, 2013)), the McGill-R-Thy1-APP model has been successful in generating many aspects of AD pathology with just a single transgene of APP combining Swedish (K670N/M671L) and Indiana (V717F) mutations (Leon *et al.*, 2010). These rats accumulate intracellular A β from just one week after birth, and the first amyloid deposits show at 6 months in a particular brain region, the subiculum. Investigations in older animals have specifically identified degenerating glutamatergic and cholinergic neurons surrounding compact, ThS-positive plaques, showing the vulnerability of these types of neurons. Furthermore, the levels of soluble trimeric A β species are greatly elevated in homozygous rats, and were found to correlate with learning and memory deficits at 3 months of age (Leon *et al.*, 2010). This observation points towards toxicity of soluble A β species, rather than mature amyloid plaques. This model thus recapitulates many features of human AD, although a subsequent study has shown that neuronal loss is in fact very limited, being largely restricted to the subiculum at 18 months of age (Ingrid *et al.*, 2015).

Another useful rat model, TgF344-AD, has been developed by expressing K670N/M671L mutant APP and PSEN1 with a deletion of exon 9 (R. M. Cohen *et al.*, 2013). At 16 months of age, the rats display widespread deposition of ThS-positive amyloid plaques in the cingulate cortex and hippocampus, as well as the striatum and cerebellum, all of which are regions affected by amyloid deposition in AD patients. The model also features intraneuronal accumulation of A β 42, and soluble oligomeric A β species have been found which correlate with significant neuronal loss; in addition the model displays inflammation and phagocytosis of neuronal debris. At the behavioural level, the rats show defects in various assays, and these progress with age following the development of the observed pathology. In contrast to most APP/PSEN based mouse models, this rat model displays tau pathology consisting of Gallyas silver stained tangles both in vicinity of the amyloid plaques, and also in regions devoid of plaques, as observed in human AD (Braak and Braak, 1991). The presence of tau tangles is critical to study the interplay between A β and tau pathology, which is still poorly understood, as outlined in the next sections.

3.2 Tau models

Yeast models

Yeast models have been employed to compare the *in vivo* aggregation propensities of various tau isoforms and disease-associated mutations, as well as the role of specific phosphorylation sites (**Table 2**). Upon expression of the human tau isoforms 2N3R and 2N4R in yeast, a small fraction of both protein constructs accumulates in the sarkosyl-insoluble aggregate fraction, with the 2N4R isoform being more extensively phosphorylated than the 2N3R isoform (Vandebroek *et al.*, 2005). Comparison of a set of six familial point mutants in the 2N4R isoform showed that all become hyperphosphorylated, and accumulate more strongly in the sarkosyl-insoluble fraction than the wild-type protein (Vanhelmont *et al.*, 2010). Purified hyperphosphorylated, soluble wild-type tau quickly forms structured filaments *in vitro*, and is able to seed tau isolated from transgenic yeast cells (Vandebroek *et al.*, 2005). The P301L tau variant found in FTDP-17 patients, purified from yeast, has been shown to aggregate *in vitro* when presented with a microtubule surface as a substrate, in contrast to wild-type tau-4R (Vandebroek *et al.*, 2006). Although yeast models have thus confirmed key aspects of tau phosphorylation and aggregation, no toxicity has been observed in any of the reported expression systems as judged from the growth rates of the cells (Vandebroek *et al.*, 2005; De Vos *et al.*, 2011).

Worm models

The first *C. elegans* models of tauopathy have been based on the overexpression of a construct comprising human tau 1N4R in nerve cells (Kraemer *et al.*, 2003). In addition to that expressing the wild-type protein, lines expressing the mutants P301L and V337M, which are associated with Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), have been generated, all resulting in an uncoordinated phenotype with reduced thrashing rates and shortened lifespan. Tau becomes phosphorylated at the majority of known sites in these models, and it accumulates in the detergent-soluble fraction as the worms age, as well as in the detergent-insoluble fraction in the case of the mutant lines. In addition to presynaptic defects in cholinergic transmission, degeneration of GABAergic axons has been observed. However, tau was found to be diffusely localised in the axons, and only the V337M variant showed clear deposits in electron micrographs. This mutant line contains the highest level of insoluble tau, and displays the strongest phenotype, but the results obtained on wild-type and P301L tau suggest that the formation of deposits is not required for neurodegeneration (Kraemer *et al.*, 2003).

A study in which the 3R and 4R isoforms of wild-type tau, and the 4R isoforms of P301L and R406W were expressed specifically in the six mechanosensory neurons of *C. elegans* has largely corroborated these findings (Miyasaka *et al.*, 2005). The touch response is only mildly affected in worms expressing wild-type tau, but declines strongly with age in the mutant strains, both of which show corresponding signs of neurodegeneration. Ultrastructural examination by EM has shown accumulation of tau in the cell bodies and neuronal processes with concomitant loss of microtubules, yet no evidence for fibrillar aggregates was found.

Expression of different tau variants with increased or decreased aggregation propensities has, however, highlighted the relevance of toxic protein aggregates in the *C. elegans* models. Co-expression of V337M tau with a short aggregation-prone tau fragment, F3ΔK280, leads to the formation of ThS-positive inclusions, and increased accumulation of full-length tau in the detergent-soluble and detergent-insoluble fractions during ageing compared to the strain expressing V337M tau alone (Fatouros *et al.*, 2012). Concomitantly, the locomotory phenotype is more severe, the neurons display more morphological abnormalities, and the transport of mitochondria is perturbed. The introduction of mutations predicted to prevent the formation of β -strands in the F3ΔK280 fragment has the opposite effect, and in addition compounds known to inhibit tau aggregation have been shown to reduce the accumulation of insoluble tau and partially restore the locomotory phenotype of the strain expressing the aggregation-prone fragment (Fatouros *et al.*, 2012).

Fly models

The first observation of tau toxicity in flies was serendipitous, when a reporter construct consisting of *Drosophila* tau tagged with GFP was expressed in sensory neurons with the aim to study axonal development. Morphological defects were observed, providing the first demonstration of tau toxicity in the fly, and similar results were obtained upon expression of human tau (Williams, Tyrer and Shepherd, 2000). Since then, *Drosophila* has been used extensively to study tau aggregation and toxicity, as well as the effects of phosphorylation by different kinases (see (Sun and Chen, 2014) for a more complete overview, and **Table 4** for the models here discussed). As in yeast and *C. elegans*, the effects of FTDP-17 mutations have also been examined, for example in models expressing human wild-type and R406W tau forms throughout the nervous system (Wittmann *et al.*, 2001). Although both of these strains display a reduction in lifespan, the phenotype is stronger for the R406W mutant, and severe neurodegeneration is evident from vacuolar structures in the cortex. Both the wild-type and R406W tau forms are recognised by antibodies specific to pathogenic phosphorylation and misfolded conformations of tau, but evidence for fibrillar structures has not been found in investigations by EM.

Co-expression of the *Drosophila* homologue of the kinase GSK-3 β with full-length tau in the fly eye does result in the characteristic flame-shaped aggregates resembling those found in human tauopathies (Jackson *et al.*, 2002). The antibody AT100, which is specific for phosphorylated tau in tangles, recognises tau only when co-expressed with GSK-3 β in the fly. Co-expression of the kinase results in fibrillar aggregates with the dimensions of human paired helical filaments (PHFs) and straight filaments (SFs) as seen by EM, and exacerbates neurodegeneration compared to the expression of tau alone (Jackson *et al.*, 2002). In agreement with the toxic effects of phosphorylation by GSK-3 β , inhibition of the kinase restores the locomotory phenotype in a model expressing human wild-type tau 0N3R in motor neurons (Mudher *et al.*, 2004). Another kinase, PAR-1, has been shown to phosphorylate tau upstream of GSK-3 β , and its overexpression exacerbates the rough eye phenotype of the R406W model, but in the absence of visible tau aggregates (Nishimura, Yang and Lu, 2004).

In certain tauopathies, fibrillary tangles occur in glia in addition to neurons (Lee, Goedert and Trojanowski, 2001). A *Drosophila* model in which human 0N4R tau is expressed specifically in adult glia shows a reduction in lifespan and the presence of apoptotic signals in glia and, by non-cell autonomous mechanisms, in cholinergic neurons (Colodner and Feany, 2010). In aged flies, tau is found in sarkosyl-insoluble fractions, which contain straight fibrils as shown by EM studies. Immunohistochemistry has confirmed that the glia contain inclusions consisting of phosphorylated tau, which are furthermore positive for ThS and Bielschowsky silver staining, and contain fibrillar structures in EM. Expression of tau in both glia and

neurons leads to increased toxicity compared to that in neurons alone, as measured by the number of apoptotic cells, indicating the importance of glial tau in these neurodegenerative disorders.

In a recent study, a comparison of a panel of *Drosophila* models expressing wild-type tau and five FTDP-17-linked mutations at similar expression levels has further strengthened the correlation between tau phosphorylation and neurotoxicity (Bardai *et al.*, 2018). Mutants that display the strongest aggregation, indicated by the number of inclusions *in vivo* and ThS fluorescence of the corresponding recombinant proteins *in vitro*, show the least caspase activation and induction of stress signalling, demonstrating that mature aggregates do not cause significant toxicity. The mutants generating the highest toxicity, on the other hand, have the highest phosphorylation levels and show increased F-actin accumulation, suggesting a correlation between actin stabilisation and toxicity (Bardai *et al.*, 2018).

Rodent models

As in the case of APP-based mouse models, initial rodent models overexpressing human wild-type tau constructs have not displayed clear behavioural phenotypes, nor produced significant numbers of tau tangles, which is why most models have been generated using tau with mutations found in FTDP-17 (reviewed in (Hutton *et al.*, 2001; Lee, Kenyon and Trojanowski, 2005)). An overview of about 30 current transgenic mouse models of tau is available on www.alzforum.org; we highlight here those that are commonly used, or that have led to particularly interesting insights (**Table 5**).

One of the few successful models based on wild-type tau is the htau model, a cross of a strain expressing human tau, including all regulatory regions and leading to all splicing variants, with a knock-out strain for endogenous mouse tau (Andorfer *et al.*, 2003). Expression of the human construct by itself does not lead to clear tau pathology (Duff *et al.*, 2000), suggesting that the interplay between mouse (which only occurs as 4R) and human isoforms is important. In the htau model, tau was observed to be correctly localised in the axons in 6-week-old animals, but tau staining was found to shift towards the cell bodies as the animals age, accompanied by increased phosphorylation at particular sites. Beyond 9 months, tau aggregation was inferred from immunohistochemistry and immuno-EM, and the sarkosyl-insoluble fraction was found to contain PHFs with similar dimensions as the ones found in AD brains (Andorfer *et al.*, 2003). The mice suffer significant neuronal loss at old age, between 8 and 18 months, but the majority of dying neurons does not appear to contain fibrillar aggregates (Andorfer *et al.*, 2005). However, memory impairments and synaptic defects occur at 12 months, but not at 4 months, indicating that tau pathology does need to develop to a certain stage to cause damage (Polydoro *et al.*, 2009).

A more recent model based on wild-type tau, Tau4RTg2652, has been generated to overexpress high levels of 1N4R wild-type tau, driven by the Thy1.2 promoter, resulting in approximately 12-fold overexpression relative to endogenous mouse tau (Wheeler *et al.*, 2015). In this model, misfolded and phosphorylated tau is detectable by various antibodies in the forebrain, amygdala, hippocampus and brainstem even at 3 months, but tangles are not observed by ThS and Gallyas staining even at 24 months of age. The levels of oligomeric tau as recognised by the TOC1 antibody are relatively low, but abnormal axon morphologies and dystrophic neurites have been nevertheless identified throughout the brain. The mice show a slight motor phenotype, but the most obvious behavioural phenotype is an impairment in spatial memory as assessed in the Barnes maze test. Altogether, these data suggest that pre-tangle tau, consisting of altered conformations and phosphorylation states, but not oligomers or fibrils, could be sufficient to drive neuronal dysfunction. Both the tau pathology and the behavioural phenotype remain stable from 3 months onwards, which may make this model suitable for rapid studies of the early stages of sporadic tauopathies, including AD.

Most other existing tau models are based on tau mutations linked to FTDP-17, which are not directly related to AD, but provide strong phenotypes, which are useful for fundamental studies of tau pathology, as well as for preclinical trials targeted at halting tau toxicity. The JNPL3 mouse, overexpressing tau 0N4R with the P301L mutation from the PrP promoter, shows impaired motor function and abundant tau tangles in several regions of the brain as well as the spinal cord; the latter are, however, typically not associated with AD (Lewis *et al.*, 2000). The tau accumulations are recognised by a range of conformational and phospho-specific antibodies, and are positive for Congo red, ThS and other staining agents such as Gallyas silver. Different morphologies have been found, including the flame-shaped tangles characteristic of AD, but also aggregates resembling Pick bodies and small, dense inclusions similar to those found in FTDP-17. EM studies have confirmed the presence of fibrils, and sarkosyl-insoluble protein has been found in extracts of brain and spinal cord from aged JNPL3 mice, but not from control lines expressing wild-type human tau.

In another mouse model, known as pR5, the P301L mutant is expressed in the context of the longest tau isoform 2N4R under the control of the Thy1.2 promoter (J. Götz *et al.*, 2001). In this model, tau also accumulates in various brain regions, and forms tangles stained by ThS and Gallyas silver in e.g. the spinal cord and layers 5 and 6 of the cortex. Reactive astrocytes and apoptotic neurons have been found in the same regions. Other neurons, including pyramidal cells in the hippocampus, show granular tau accumulation that is recognised by a subset of phospho- and conformation-specific antibodies. The sarkosyl-insoluble fraction of brain extracts contains fibrils as seen by EM, but they are shorter and narrower than fibrils obtained from AD patient material. This is in line with the observation that P301L

fibrils from FTDP-17 patients have a different morphology from that of V337M FTDP-17 fibrils or wild-type AD fibrils (Spillantini, R A Crowther, *et al.*, 1998), suggesting that the point mutations can significantly affect fibril structure. Furthermore, the strong overexpression in mouse models may give rise to shorter fibrils than those observed in AD, because the likelihood of primary nucleation is higher.

The rTg4510 model has also been designed to express P301L tau (0N4R), in this case using an elegant expression system that can be inhibited with doxycycline (Ramsden *et al.*, 2005; SantaCruz *et al.*, 2005). The Ca²⁺/calmodulin kinase II (CaMKII) promoter has been used to drive expression specifically in the forebrain, where the first tau tangles appear at 2.5 months of age, leading up to extensive tau pathology and increasing atrophy in the cortex and hippocampus from 5 months onwards (Ramsden *et al.*, 2005). Misfolded tau conformations and phosphorylation also develop with age, as indicated using various antibodies, and the main tau species shifts from the soluble 55 kDa form at 2.5 months to the sarkosyl-insoluble, extensively phosphorylated 64 kDa-species at 8.5 months. At the ultrastructural level, straight filaments have been observed in EM on fixed tissue samples from 10-month-old animals, which occasionally adopt a typical herring bone pattern which has also been observed in deposits in Pick's disease. At the behavioural level, spatial memory in the Morris water maze test is impaired from 4 months of age, and locomotory deficits have been observed in 10-month old mice (Ramsden *et al.*, 2005).

The possibility of lowering tau expression in this model has led to interesting insights into the relationship between tau pathology and toxicity (SantaCruz *et al.*, 2005). Suppressing tau expression at 2.5 months causes the pathology to be halted. In contrast, when doxycycline treatment is given at 4 months or later, tau phosphorylation and accumulation in the insoluble fraction proceed, and numbers of tau tangles grow similarly as in untreated animals. Given the significant reduction in tau mRNA, this suggests that the soluble species that were already present can develop into pathological tau. However, brain atrophy and memory defects were found to be largely restored if the doxycycline treatment was sufficiently long, demonstrating that the presence of tau tangles is not directly correlated with the disruption of cognitive function (SantaCruz *et al.*, 2005).

A model expressing tau 1N4R with the P301S mutation, PS19, shows a strong motor phenotype which presents difficulties for behavioural studies, yet it has been a useful model because of its robust tau pathology and evident neurodegeneration (Yoshiyama *et al.*, 2007). Phosphorylated tau has been shown to accumulate rapidly, and increased levels of insoluble tau have been observed during ageing. At 6 months of age, flame-shaped tau tangles and globular aggregates, as well as thread-like structures, can be visualised in the hippocampus, amygdala and spinal cord by Gallyas silver staining, and by staining with ThS and

Congo red. Activated microglia and astrocytes, as well as neuronal inflammatory markers, develop from 3 months onward. Tau pathology and inflammation precede large scale neurodegeneration, which is observed from 9 months onwards in the hippocampus and other brain regions. However, hippocampal synapses are already lost by 3 months of age, preceding the appearance of tau tangles. As such, the results obtained from this model add to the notion that neurodegeneration is initiated before mature protein aggregates are formed.

The Δ K280 mutation, which is known from a FTDP-17 case, is directly correlated with the aggregation propensity of tau, being located in a segment of the protein that forms a β -strand in the fibril structure. Mouse models based on this pro-aggregation mutant have been generated in parallel with an anti-aggregation mutant consisting of Δ K280 combined with two proline mutations (I277P and I308P), which strongly inhibit β -strand formation (Eckermann *et al.*, 2007; Mocanu *et al.*, 2008). These mutants have been expressed in the context of full-length human tau 2N4R (Eckermann *et al.*, 2007), and in a short construct comprising only the repeat domain of tau (Mocanu *et al.*, 2008) in two respective studies, leading to very similar results. The pro-aggregation mutant adopts a misfolded conformation that is recognised by the MC1 antibody, becomes phosphorylated at several sites and is partially found in the sarkosyl-insoluble fraction, whereas the anti-aggregation mutant does not display these features. Furthermore, the pro-aggregation mutant has been shown to induce co-aggregation of endogenous mouse tau, which co-localises with the exogenous human tau in the same filaments extracted from the mouse brain. In both studies, quantification from electron micrographs has shown that the pro-aggregation mutant causes more synaptic loss than the anti-aggregation mutant. The strong correlation between *in vitro* and *in vivo* aggregation propensities is furthermore underscored by the fact that the truncated construct leads to Gallyas-positive tau tangles much earlier than full-length tau, in line with a protective effect of the flanking regions on tau aggregation observed *in vitro*. These models also make use of the regulatable expression system, and switching off tau expression has been shown largely to reverse tau pathology (Eckermann *et al.*, 2007; Mocanu *et al.*, 2008), in contrast to the earlier study which reported that tau tangles continue to develop after reducing transgene expression, although memory function recovered (SantaCruz *et al.*, 2005). However, phosphorylation and aggregation of endogenous mouse tau persist in the pro-aggregation model, possibly seeded by the pathological human tau expressed prior to switching off the transgene (Mocanu *et al.*, 2008).

More recently, mouse models have been generated expressing tau with the A152T mutation, which has been found to increase the risk of FTD as well as AD. In one model, the mutation has been introduced in the context of full length tau 2N4R (Decker *et al.*, 2016; Sydow *et al.*, 2016), whereas another model that was simultaneously reported is based on the expression of tau 1N4R (Maeda *et al.*, 2016). Both models display reactive microglia and astrocytes and neuronal hyperexcitability, with deficits in behavioural assays

and neuronal loss occurring at later age. The model expressing A152T tau 2N4R was shown to develop ThS and Gallyas silver positive tau tangles starting from 3 months of age (Sydow *et al.*, 2016), but in the model expressing the 1N4R isoform, the misfolded tau was found to remain largely soluble (Maeda *et al.*, 2016). Thus, soluble A152T tau oligomers may be responsible for the overlapping phenotypes of these two models.

Rat transgenic models have been used less extensively than mouse to study human tauopathies. A rat model expressing full-length tau with the P301L mutation displays tau phosphorylation and accumulation in the sarkosyl-insoluble fraction, but no tangles, nor behavioural abnormalities (Korhonen *et al.*, 2011). Models based on truncated 4R and 3R isoforms (residues 151-391), which are thought to play a role in AD, do display full tau pathology including the presence of phosphorylated, misfolded and sarkosyl-insoluble tau, and ThS and Congo red positive tangles, but without overt neuronal loss (Zilka *et al.*, 2006; Koson *et al.*, 2008; Filipcik *et al.*, 2012).

3.3 Models combining A β and tau

Worm models

The link between A β and tau pathologies in AD has remained incompletely understood, in part because animal models have not been particularly successful in recapitulating both features of the disease. Crossing *C. elegans* strains expressing A β 42 and an aggregation-prone tau mutant in neurons was shown to lead to an exacerbation of the toxic phenotype in terms of lifespan, viability of offspring, chemotaxis and other behavioural assays (Wang *et al.*, 2018). Conversely, crossing an anti-aggregation tau variant and an A β 42 strain has not led to a significantly different phenotype compared to A β 42 alone. The total aggregation load as seen from Congo red staining was found to be much increased in the double transgenic line with the pro-aggregation tau mutant, and signs of neurodegeneration and neuronal loss were observed, but a mechanistic link between A β 42 and tau toxicity has not been revealed from these studies.

Fly models

Co-expression of A β 42 has been shown to exacerbate the phenotype of wild-type human tau transgenic flies, manifesting in disrupted axonal transport, abnormal axonal morphology, impaired motor function, and shortened lifespan (Folwell *et al.*, 2010). This effect has been suggested to be mediated by increased tau phosphorylation in the presence of A β 42, but the responsible kinases have so far not been identified. In

an inducible fly model expressing A β 42 with the Arctic mutation (E22G), the *Drosophila* kinase GSK-3 β is activated, and A β 42-mediated toxicity is reduced when endogenous fly tau is removed (Sofola *et al.*, 2010). However, differences in the phosphorylation of *Drosophila* tau in the presence or absence of Arctic A β 42 are below detection limits. Thus, the link between A β 42 and tau phosphorylation remains unclear from the *Drosophila* studies.

Rodent models

APP/PSEN-based rodent models have largely failed to induce significant tau pathology, although tau knock-out models have demonstrated that the presence of tau is required for A β -related toxicity to occur fully (Roberson *et al.*, 2007; Leroy *et al.*, 2012). To induce both A β and tau pathology, however, it has been necessary to develop double or triple transgenic models overproducing combinations of human APP, PSEN1/2 and tau, often with mutations that do not occur simultaneously in AD patients. Nevertheless, these models have proved to be useful for understanding the interactions between A β and tau pathology in AD.

The first transgenic mouse model combining APP with tau has been the TAPP mouse, resulting from a cross of Tg2576, which expresses APP containing the Swedish mutation (K670N/M671L) with the JNPL3 mouse, which expresses the P301L tau variant (Lewis *et al.*, 2001). The number of tau tangles is highly increased in the TAPP mouse compared to the original JNPL3 mouse, and tangles are found in brain regions that are unaffected in the JNPL3 model, including the hippocampus, amygdala and entorhinal cortex. Other regions that are affected by tau pathology in the JNPL3 mouse, such as the hindbrain and spinal cord, do not show an increase. A β deposition is, however, not changed compared to the Tg2576 model, in line with the amyloid cascade hypothesis, according to which A β aggregation occurs upstream of tau pathology (Selkoe and Hardy, 2016). Despite the clear link, tau tangles are not identified in close vicinity of amyloid plaques, indicating that other mechanisms, perhaps mediated by intracellular A β , may play a role. These results have been confirmed by an independent study in which A β 42 fibrils were injected in the pR5 mouse model which also expresses tau P301L, resulting in a 5-fold increase in tau tangles (J Götz *et al.*, 2001). In this experiment, the main increase in tau tangles was found in neurons in the amygdala, which are distant but projecting towards the injection site, raising the possibility that retrograde transport of A β 42 can promote tau tangle formation.

A triple transgenic mouse model, the 3xTg-AD mouse, has been created by co-injecting constructs of the Swedish APP variant (K670N/M671L) and the P301L tau variant into embryos carrying the M146V PSEN1 variant (Oddo *et al.*, 2003). This model has been used extensively in AD research because the temporal and

spatial appearance of amyloid plaques and tau tangles resembles that observed in the human disease. Antibody staining for A β is first observed intracellularly at 3-4 months of age, mostly in the neocortex and subsequently in the hippocampus. Plaques develop progressively in the same regions between 6 and 12 months. Tau pathology is initiated later and follows a different spatial pattern, first accumulating in pyramidal neurons in the hippocampus, followed by cortical regions. Aggregated tau can be recognised by conformation-specific and phospho-specific antibodies, as well as Gallyas silver and ThS staining. Although this sequence of events does not prove causality between A β and tau aggregation, both proteins have been found to co-localise within the same neurons, suggesting a role for intracellular A β in triggering tau pathology. Synaptic defects are observed in the hippocampus at 6 months in this model, which correlates with the presence of intracellular A β .

Models based on the PS19 line, expressing the P301S tau variant, have largely confirmed these findings. Crossing this line with the PDAPP mouse or the 5xFAD line has resulted in exacerbated tau pathology, whereas A β deposition is not significantly affected (Hurtado *et al.*, 2010; Saul *et al.*, 2013). The 5xFAD/PS19 model furthermore shows increased astrogliosis compared to the original transgenic lines, as well as increased loss of synapses and neuronal death (Saul *et al.*, 2013). The PDAPP/PS19 mice have been used for detailed analysis of the spatial progression of tau pathology, which matches to some extent the Braak staging of AD (Hurtado *et al.*, 2010). However, a limitation of the PS19 model and crosses thereof is their strong motor phenotype, which interferes with commonly used behavioural assays.

Another triple transgenic model, TauPS2APP, has resulted from crossing the PS2APP line expressing the K670N/M671L APP variant and the N141I PSEN2, with the pR5 tau P301L strain (Grueninger *et al.*, 2010). In contrast to the models described above, amyloid plaques and accumulations of phosphorylated tau develop in parallel in this model, and in the same regions of the hippocampus. Gallyas silver staining and immuno-EM confirmed that the deposits of tau are of fibrillar nature, and that structures resembling the paired helical filaments observed in AD are present within dendrites. Phosphorylation of S422 has been shown by means of highly specific antibodies to be significantly increased in the triple transgenic line, compared to the original tau model. However, the triple transgenic mice do not show signs of increased neuronal loss compared to wild-type controls, and perform similarly to the PS2APP strain in behavioural assays.

PLB1 double mice have been generated by targeted knock-in of the APP K670N/M671L/V717I and tau P301L/R406W mutants (Platt *et al.*, 2011), resulting in more moderate expression levels than in models generated by pronuclear injection, a process that leads to integration of multiple copies of the transgene.

This line has been subsequently crossed with a PSEN1 A246E strain (Borchelt *et al.*, 1997) to generate the PLB1 triple mouse model (Platt *et al.*, 2011). Despite the low levels of A β in the triple strain, intracellular staining as well as a small number of extracellular ThS and Congo red positive plaques appear at 6 months of age in the hippocampus and cortex, and the number of plaques increases during ageing. Phosphorylated tau has been identified in the same brain regions from 6 months of age, but no evidence for mature tau tangles was found. Synaptic plasticity, together with social recognition and spatial memory, start to decline at the same age. Furthermore, glucose metabolism and sleep behaviour are altered in the PLB1 triple mice, as found in patients suffering from AD. Altogether, despite some discrepancies such as the relatively fast accumulation of phosphorylated tau, this less aggressive model recapitulates many features of the early phases of human AD.

Although most studies using double and triple transgenic mice have shown a unidirectional relationship between APP/A β expression and increased tau pathology, there is an example in which amyloid deposition is increased by mutant tau expression. In that work, Tg2576 expressing APP with the K670N/M671L Swedish mutation was crossed with a mutant tau line, tau^{VLW}, containing G272V, P301L and R406W mutations (**Figure 3H**) (Pérez *et al.*, 2005; Ribé *et al.*, 2005). Not only does the expression of APP^{Swe} lead to increased tau phosphorylation and accumulation in the sarkosyl-insoluble fraction (Pérez *et al.*, 2005), but expression of the tau mutant also increases the numbers of amyloid plaques by up to 5-fold at 16 months of age (Ribé *et al.*, 2005). The double transgenic animals also suffer from more pronounced neuronal loss than either APP^{Swe} or tau^{VLW} littermates (Ribé *et al.*, 2005).

The models discussed so far have been based on tau mutations found in FTDP-17 patients, or combinations thereof, and they typically display tau pathology in the single transgenic lines, albeit increased by expression of A β . In a model combining wild-type human tau 0N3R with APP K670N/M671L/V717I and PSEN1 M146L, tau tangles are absent (Boutajangout *et al.*, 2004). However, in addition to APP-stained and ubiquitin-stained dystrophic neurites, tau-positive dystrophic neurites are also found surrounding amyloid plaques in these triple transgenic mice. In these neurites, tau is recognised by a subset of phospho-specific and conformation-specific antibodies, but is not fibrillar, as indicated from a lack of Gallyas or Congo red staining. Similar observations have been reported for the TAPP and 3xTg-AD mice, although in those models tau tangles do occur in more distant regions from the A β plaques (Lewis *et al.*, 2001; Oddo *et al.*, 2003). Similarly, in a more recent model resulting from a cross of WT human tau 4R with APP^{Swe} and PSEN1 Δ exon9, no evidence was found for the formation of tau tangles, although the mice were only examined up to 10 months of age (Jackson *et al.*, 2016). This model, however, displays increased plaque

counts and dystrophic neurites compared to the APP/PSEN1 mice, although no increase in neuronal loss or astrogliosis was observed.

In contrast, another model based on human wild-type tau does feature the development of tangles. This model is the result of a cross between animals expressing APP Δ E693 (the Osaka mutant) and a tau construct comprising 3R and 4R isoforms (Umeda *et al.*, 2014). APP Δ E693 is not associated with amyloid plaque formation, but generates high levels of A β oligomers. These oligomers, detected by the 11A1 conformational antibody, appear earlier in double transgenic mice than in the original APP line, being evident intracellularly in regions of the hippocampus and cerebral cortex at 6 months of age. Furthermore, hyperphosphorylated tau, recognised by AT8 and PHF1 antibodies, is formed more rapidly in the CA3 region of the hippocampus and in the cortex in double transgenic animals, appearing between 6 and 12 months. Tau tangles are present in the same regions as detected by Gallyas silver staining and EM from 18 months of age onwards. Loss of synapses and neuronal death in the CA3 region are also increased in the double transgenic line, as are the memory deficits observed in the Morris water maze test. Altogether, whereas other models have failed to produce the full pathology of wild-type tau isoforms, the combination of tau with APP Δ E693 has had better success in doing so. This finding demonstrates that the presence of A β plaques is not a prerequisite for tau pathology and neuronal dysfunction to occur, but A β oligomers may be responsible for triggering this cascade instead.

Taking advantage of the knock-in strategy described in Section 3.1 for the APP-based models, a recent study reports on the humanisation of the mouse *MAPT* gene (Saito *et al.*, 2019). Whereas the subcellular distribution of human tau appears normal in this model, crossing it with the APP knock-in model, APP^{NL-G-F}, results in enhanced tau phosphorylation at several sites. Tau tangles were not identified, yet these more physiological models may prove useful for further studies into the relationship between A β and tau pathology.

Finally, it is worth noting that the spreading of tau pathology by means of neuronal connectivity has received much attention in recent years (Goedert, Eisenberg and Crowther, 2017; Jucker and Walker, 2018). In a model expressing the human P301L tau variant exclusively in layer II of the entorhinal cortex, it has been shown that this protein is present in synaptically connected neurons that do not express it, and that it seeds the aggregation of endogenous mouse tau (de Calignon *et al.*, 2012). Crossing this line with the model that expresses APP^{swc} and PSEN1 Δ exon9 has led to an increased spread of misfolded tau, and to increased neuronal loss in the entorhinal cortex where tau is expressed (Pooler *et al.*, 2015). In addition, at 16 months

of age, the triple transgenic animals display more and larger A β deposits in the entorhinal and somatosensory cortices, and more dystrophic neurites surrounding the plaques.

In summary, although it has proved to be difficult to recapitulate the complex pathology and phenotype of human AD in rodent models, there has been significant progress towards this objective as a result of combining in a variety of ways transgenes for APP, presenilins and tau.

3.4 α -Synuclein models

Yeast models

The first yeast model for PD, based on the expression of human α -synuclein in *S. cerevisiae*, was reported in 2003 (**Figure 3B**) (Outeiro and Lindquist, 2003). Expression of a single copy of GFP-tagged human α -synuclein resulted in the localisation of the protein at lipid membranes, whereas expression of two copies caused it to form cytoplasmic inclusions containing lipid molecules, as shown by staining with the lipophilic dye Nile red, in addition to protein. Furthermore, a dose-dependent cytotoxicity was observed, consistent with a toxic gain of function associated with α -synuclein expression. Two familial α -synuclein mutants A30P and A53T were also investigated in this study. While the A53T mutant was found to display very similar behaviour to wild-type α -synuclein, the A30P mutant was shown to maintain a cytoplasmic distribution at single copy level, rather than accumulating at the lipid membrane, and to exhibit toxicity only when expressed from a high-copy plasmid (Outeiro and Lindquist, 2003). Subsequently, inclusions of A53T α -synuclein, either GFP-tagged or unlabelled, were shown to react with ThS, indicating an amyloid-like structure, whereas staining was not observed in cells expressing the A30P mutant (Zabrocki *et al.*, 2005). These *in vivo* models (**Table 2**) have confirmed the importance of the interactions of α -synuclein with lipid membranes, and have provided insights into the correlation between inclusion formation, amyloid-like aggregation and cytotoxicity.

Worm models

The tendency of α -synuclein to accumulate into cytoplasmic inclusions was further corroborated in *C. elegans* models (**Table 3**). A model overexpressing yellow fluorescent protein (YFP)-tagged α -synuclein in the body wall muscle cells displays inclusions from day 1 of adulthood, and these increase in number as the worms age (van Ham *et al.*, 2008). Interestingly, fluorescence recovery after photobleaching (FRAP) measurements (**Table 1**) have indicated that α -synuclein remains mobile within the inclusions, and only in

old worms (from day 11 onwards) immobile inclusions, indicative of aggregated proteins, occur (van Ham *et al.*, 2008). These results were confirmed recently by FLIM (**Table 1**), in which reduction of the fluorescence lifetime of the YFP tag, indicative of fibril formation by α -synuclein, is only observed after day 10 (Laine *et al.*, 2019). However, motility defects are observed from day 4 onwards (van Ham *et al.*, 2008), suggesting that inclusion formation generates toxicity prior to the formation of detectable amyloid-like aggregates.

C. elegans has a particular advantage for PD research in that it contains eight dopaminergic neurons that can easily be tracked. Models overexpressing wild-type and mutant α -synuclein in dopaminergic neurons have been generated in several independent studies (Lakso *et al.*, 2003; Cao *et al.*, 2005; Kuwahara *et al.*, 2006). Although inclusions have only been found in a small fraction of neurons (Lakso *et al.*, 2003; Kuwahara *et al.*, 2006), or not at all (Cao *et al.*, 2005), all studies report a loss of neuronal integrity. Expression of A53T and A30P, but not wild-type, α -synuclein was found to be associated with a loss of dendrites, in agreement with behavioural defects in food sensing, which depends on dopaminergic neurons (Kuwahara *et al.*, 2006). This phenotype has been observed in 3-day old animals (Kuwahara *et al.*, 2006), and in 4-day old animals in another study (Lakso *et al.*, 2003), suggesting that neurotoxicity occurs relatively early in the lifespan of the animals. Furthermore, these studies suggest that α -synuclein neurotoxicity is not correlated with inclusion formation, but may be caused by the presence of smaller aggregates or soluble oligomeric species of α -synuclein.

Fly models

Expression of wild-type, A53T and A30P α -synuclein in the nervous system of *Drosophila* was found to lead to the formation of age-related Lewy body-like inclusions, correlated with the loss of dopaminergic neurons and impaired climbing function (Feany and Bender, 2000). In another model expressing the same α -synuclein variants specifically in the dopaminergic neurons, neuronal loss was also observed which could be restored by overexpression of the molecular chaperone Hsp70 (Auluck *et al.*, 2002). Some controversy remains, however, about the extent of neuronal loss in fly models of PD, and other groups who used different methods have reported that the neurons might have been dysfunctional instead of lost (Pesah *et al.*, 2005; Botella, Bayersdorfer and Schneuwly, 2008). The inclusions in the original model were examined by EM to reveal mostly round aggregates consisting of a rather homogeneous core, surrounded by radiating filaments (Feany and Bender, 2000). The model also displays diffuse aggregates and neuritic threads. Perhaps surprisingly, no differences between the phenotype of the wild-type and mutant α -synuclein lines have been found in this study (Feany and Bender, 2000). The severity of the phenotype may correlate better

with the α -synuclein expression levels, given that a more recent model achieving higher expression levels in the fly brain using the Q binary expression system shows exacerbated locomotory defects and a shortened lifespan, accompanied by α -synuclein inclusions that are mostly ubiquitin-positive, and vacuolar neurodegeneration (Ordonez, Lee and Feany, 2018).

The relationship between α -synuclein aggregation and neurotoxicity has been further addressed by models expressing truncated or modified forms of the protein. A construct lacking part of the non-amyloid component (NAC) domain, which is essential for α -synuclein aggregation (**Figure 2C**), does not induce either inclusion formation or degeneration of dopaminergic neurons, whereas a more aggregation-prone C-terminal truncation mutant shows increased deposition of proteins resistant to proteinase K (PK), as well as higher toxicity, supporting a direct correlation between α -synuclein aggregation and toxicity (Periquet *et al.*, 2007). Notably, analysis by western blot has indicated that the levels of soluble oligomers are decreased in the NAC deletion flies, and increased in the C-terminal mutant line, leaving the question open as to whether the PK resistant species or the soluble oligomers are responsible for toxicity (Periquet *et al.*, 2007).

Other studies have shown that the presence of large inclusions does not correlate with toxicity, and may even be protective. When phosphorylation of S129 is blocked by the mutation of serine to alanine, more inclusions are formed, correlating with more PK-resistant protein, but a reduced loss of dopaminergic neurons (Chen and Feany, 2005). How this observation relates to PD in humans is unclear, as S129-phosphorylated α -synuclein is found in Lewy bodies (Fujiwara *et al.*, 2002). However, a subsequent study has shown that S129 phosphorylation increases oligomer formation, explaining the exacerbated phenotype in the fly model, whereas phosphorylation at Y215 is protective, as blocking this modification results in increased oligomer levels and toxicity (Chen *et al.*, 2009).

The role of α -synuclein oligomers has been further addressed by designing proline mutants that prevent β -strand formation in α -synuclein (Karpinar *et al.*, 2009). These mutations have been found to inhibit fibril formation *in vitro*, yet increase the generation of oligomeric species. When expressed in *C. elegans* vulval muscles, wild-type α -synuclein-YFP forms elongated structures, whereas a triple proline mutant shows a diffuse localisation consistent with the absence of large aggregates (Karpinar *et al.*, 2009). However, the triple proline mutant shows increased toxicity in both worm and fly models when expressed in dopaminergic neurons, supporting a role for α -synuclein oligomers in neurotoxicity (Karpinar *et al.*, 2009).

Rodent and primate models

As described above in the context of AD models, it has been challenging to develop a rodent model that recapitulates the main characteristic features of PD, including Lewy body pathology, specific loss of dopaminergic neurons in the substantia nigra, and loss of motor function. A complication arises from the fact that mice and rats express their endogenous forms of α -synuclein, which differ from the human form at seven residues, one of the differences being the A53T substitution, which in humans is associated with familial PD. Here, we discuss rodent models based on the transgenic expression of human α -synuclein, focusing on its misfolding and aggregation and the associated phenotypes (**Table 5**). For a more complete description, see (Fernagut and Chesselet, 2004; Dehay and Fernagut, 2016; Visanji *et al.*, 2016), and for a discussion on models designed to study the spreading of α -synuclein pathology from cell-to-cell in a prion-like fashion, which is thought to play a role in synucleinopathies, we refer to (Dehay *et al.*, 2016).

The first transgenic mouse model expressing human wild-type α -synuclein, which used the PDGF- β promoter, showed accumulation of the protein in several brain regions, including the neocortex, hippocampus and substantia nigra (Masliah *et al.*, 2000). However, these inclusions did not resemble Lewy bodies, appearing in the nucleus as well as the cytoplasm, being associated with the ER, having a less well-defined shape, and consisting of granular rather than fibrillar material. Use of the Thy1 promoter led to a different spatial expression pattern of wild-type α -synuclein (Rockenstein *et al.*, 2002), and extensive characterisation of this model, termed Line 61, has revealed PK-resistant inclusions, phosphorylation of S129, reduced dopamine levels, and progressive PD-like symptoms including olfactory deficits and locomotory impairments, but no substantial neuronal loss (Chesselet *et al.*, 2012). A model expressing A53T α -synuclein under control of the strong PrP promoter showed insoluble, fibrillar protein and inclusions that stain for ThS, in contrast to the wild-type protein (Giasson *et al.*, 2002). At 8 months of age, the A53T mice start to develop motor defects, whereas wild-type α -synuclein transgenic mice are unaffected. However, the A53T phenotype is not associated with a loss of dopaminergic neurons in the substantia nigra in this model (Giasson *et al.*, 2002).

Conversely, other transgenic models led to degeneration of dopaminergic neurons in the substantia nigra but without the presence of observable inclusions, as exemplified by models expressing a combined A30P + A53T mutant (Richfield *et al.*, 2002; Thiruchelvam *et al.*, 2004), and a C-terminally truncated construct (Wakamatsu *et al.*, 2008), respectively. In a model expressing wild-type human α -synuclein from a bacterial artificial chromosome (BAC) construct that includes the native human regulatory sequences, defects in dopaminergic neurotransmission are accompanied by the presence of high molecular weight

soluble species, although PK-resistant aggregates have not been identified, suggesting that toxicity may arise from intermediate species along the aggregation pathway (Janezic *et al.*, 2013). A mouse model with inducible A53T α -synuclein expression similarly shows high molecular weight species on western blots in the absence of large inclusions, which correlate with perturbations in the Golgi apparatus in midbrain dopaminergic neurons, as well as changes in lysosomal and autophagic markers (Lin *et al.*, 2012).

A recent mouse model was created following the hypothesis that native α -synuclein can occur as folded tetramers, such that a shift to the monomeric conformation puts the protein at risk of aggregation (Bartels, Choi and Selkoe, 2011; Dettmer *et al.*, 2015). Overexpression of a triple mutant thought to disrupt the tetramers, referred to as 3K because of the three E-to-K substitutions, gives rise to a strong motor phenotype, selective degeneration of dopaminergic neurons in the substantia nigra, and abnormal accumulation of α -synuclein co-localising with vesicles (Nuber *et al.*, 2018). When compared at similar expression levels, the phenotype of the 3K mutant mice is stronger than that of a model expressing a BAC construct with the single E46K mutant that is found in some cases of familial PD. In addition, 3K is present in C-terminally truncated and S129 phosphorylated forms, and in older mice fibrillar material is present in the α -synuclein/vesicle assemblies (Nuber *et al.*, 2018), reminiscent of recent investigations showing that Lewy bodies contain vesicular structures (Shahmoradian *et al.*, 2019)[Mahul-Mellier, Anne-Laure, et al. "The process of Lewy body formation, rather than simply α -synuclein fibrillization, is one of the major drivers of neurodegeneration." *Proceedings of the National Academy of Sciences* 117.9 (2020): 4971-4982.].

Applying the BAC approach to create transgenic rats has led to a model that also recapitulates these and other features of human PD (Nuber *et al.*, 2013). Full-length as well as C-terminally truncated insoluble α -synuclein accumulate as the rats age, following a similar development as that observed in the Braak stages of PD (Braak *et al.*, 2003). PK-resistant inclusions are found in several brain regions including the substantia nigra, and the presence of aggregated protein has been further confirmed by dot blots using the FILA-1 antibody which recognises α -synuclein oligomers and fibrils but not monomers (Nuber *et al.*, 2013). Electron micrographs have shown inclusions containing lysosomes and lipid droplets, as well as accumulated vesicles at synaptic termini. In addition to the accumulation of α -synuclein, the phenotype of this rat model also recapitulates certain aspects of the course of PD in humans, with early changes in avoidance and novelty-seeking behaviour and defects in the sense of smell, followed by later motor symptoms, which correlate with the loss of dopaminergic neurons (Nuber *et al.*, 2013).

Injection of α -synuclein constructs in lentiviral (LV) or recombinant adeno-associated viral (rAAV) vectors has been used as an alternative approach to generate high local concentrations of the protein in the substantia nigra and to induce rapid neurodegeneration specifically in this region. The majority of viral vector rat and mouse models displays α -synuclein accumulation in the cytoplasm as well as in dystrophic neurites, yet does not feature Lewy body-like pathology (Kirik *et al.*, 2002; Lo Bianco *et al.*, 2002; Chung *et al.*, 2009; Dehay and Fernagut, 2016). However, PK-resistant α -synuclein and small ThS-positive inclusions have been observed in a rat rAAV model (da Silveira *et al.*, 2009). α -Synuclein accumulates close to the nucleus, the ER and Golgi in this model, and some fibrillar structures were observed in electron micrographs to co-localise with lysosomes and other vesicles. Interestingly, the number of ThS-positive inclusions was significantly increased when using the S129A α -synuclein mutation, which cannot be phosphorylated (da Silveira *et al.*, 2009), an observation analogous to the results obtained in the *Drosophila* model (Chen and Feany, 2005). On the other hand, the phosphomimetic S129D mutation displays fewer, but larger ThS positive aggregates, whereas no difference in toxicity was found between the different phosphorylation states (da Silveira *et al.*, 2009). However, another study using rat rAAV models reports that the non-phosphorylatable S129A mutant causes more dopaminergic neurodegeneration, whereas S129D forms more inclusions, although the latter have not been examined ultrastructurally (Gorbatyuk *et al.*, 2008). The precise role of S129 phosphorylation in α -synuclein aggregation and toxicity thus remains to be determined (Sato, Kato and Arawaka, 2013).

Viral vector models have provided a unique means to assess the effect of varying α -synuclein concentrations on its aggregation and the time course of neurodegeneration following injection. The appearance of ThS positive α -synuclein A53T inclusions is dose-dependent and time-dependent in rats, and correlates with dopaminergic dysfunction (Van der Perren *et al.*, 2015). Western blots have confirmed that insoluble and phosphorylated α -synuclein accumulates over time, and that high molecular weight oligomeric α -synuclein species are apparent at later time points after injection (Van der Perren *et al.*, 2015). Using the same type of viral vector, a mouse model has also been established that displays Lewy body-like pathology and degeneration of dopaminergic neurons, phosphorylation of S129 and impairments in motor behaviour (Oliveras-Salv   *et al.*, 2013). Both wild-type and A53T α -synuclein lead to similar levels of protein accumulation and loss of dopaminergic neurons using this approach. Another type of optimised rAAV vector has also been employed successfully in mice to express A53T α -synuclein locally leading to a robust phenotype, including the loss of dopaminergic neurons and reduced dopamine levels, accompanied by the formation of PK-resistant α -synuclein inclusions in cell bodies and dystrophic neurites, resembling PD pathology (Ip *et al.*, 2017).

The success with rAAV vectors in rodents has led to the development of non-human primate models using the same technology, resulting in a marmoset model that features α -synuclein accumulation in the cytoplasm and neurites, as well as partial dopaminergic neurodegeneration and motor symptoms (Kirik *et al.*, 2003). In a subsequent study, the monkeys were followed over a longer period of up to a year, leading to the discovery of ubiquitin-positive inclusions, which also occur in human PD (Eslamboli *et al.*, 2007). Furthermore, animals injected with the A53T α -synuclein construct show stronger neurodegeneration compared to the wild-type protein and a worsened phenotype at later time points, potentially making this a useful model to test therapeutic strategies.

4. Identification of biological pathways using *in vivo* models

Several current disease models are successful in recapitulating key aspects of protein misfolding and aggregation, and exhibit toxic phenotypes, making them useful to interrogate disease mechanisms by identifying the perturbed cellular pathways. Excellent tools are available for commonly used model organisms to probe these questions at the systems level by means of genomic, transcriptomic and proteomic studies.

Simple model organisms, including yeast, worms and flies, are amenable to large screens to identify genetic modifiers of protein aggregation and the associated toxicity. In addition, transcriptomics and proteomics can be applied to any organism of which the RNA or protein content can be extracted, to inform on the dysregulation of cellular pathways upon expression of the disease-related gene. Improvements in sequencing technologies have recently increased dramatically the scope of transcriptomics studies, which were previously limited to microarray analysis on a predefined set of genes, whereas genome-wide expression levels can now be determined routinely with high sensitivity and few artefacts using next generation sequencing methods such as RNAseq (Wang, Gerstein and Snyder, 2009; Breschi, Gingeras and Guigó, 2017). Similarly, developments in mass spectrometry and methods for data analysis have greatly enhanced the possibilities of performing comparative and quantitative proteomics to identify changes in protein abundance as well as posttranslational modifications (Bantscheff *et al.*, 2012; Feng, Cappelletti and Picotti, 2017; Schubert *et al.*, 2017). Not only have mass spectrometers improved in terms of sensitivity and resolution, but the opportunities for quantitative analysis of animal and human samples have expanded significantly by chemical labelling approaches such as tandem mass tags (TMTs) and isobaric tags for absolute and relative quantification (iTRAQ). Alternatively, label-free approaches based on counting the

number of peptide-to-spectrum matches for each protein have gained popularity. In addition, dedicated proteomic workflows exist that inform on protein-protein interactions and on protein conformations, although these methods are currently largely restricted to unicellular organisms and cultured cells (Minde, Dunker and Lilley, 2017).

In this section, we highlight the key pathways and genes that have been identified using these various technologies in relation to protein misfolding in the organismal models of AD and PD (**Tables 2-5**).

4.1 Biological pathways in Alzheimer's disease models

Yeast models

Of the *in vivo* models described here, yeast is perhaps the most highly amenable to systematic analysis at the level of the genome, transcriptome and proteome. Extensive toolkits are available, and profiling of genetic interactions, mRNA expression levels, protein levels, metabolite levels, biological effects of small molecules, and phenotypic responses can be performed on a large scale fairly readily (Khurana and Lindquist, 2010). Although yeast does have a nervous system, or the secretase activity required for APP processing, it has been used to identify conserved genetic factors involved in A β toxicity (Treusch *et al.*, 2011). The most potent suppressors of such toxicity were found to be genes involved in endocytosis, including the yeast homologue of *PICALM*, which is a known risk factor for AD as identified by genome wide association studies (GWAS). A number of these hits have been confirmed in a *C. elegans* model expressing A β 42 in glutamatergic neurons (Treusch *et al.*, 2011). Microarray analysis revealed the induction of ER stress and downregulation of mitochondrial processes upon expression of A β 42 targeted to the secretory pathway (Chen *et al.*, 2017).

Worm models

The main power of *C. elegans* in genetic screens is the ease with which RNA interference (RNAi) can be performed, simply by feeding the worms with bacteria expressing the relevant double stranded RNA (dsRNA). Libraries targeting 86% of all the annotated *C. elegans* genes are available and can be routinely screened if an appropriate read-out is available (Sin, Michels and Nollen, 2014). Performing RNAi by feeding is very efficient for certain tissues, such as intestinal and muscle cells, but works less well in neurons. With respect to A β toxicity, *C. elegans* has been used for an RNAi screen specifically targeted at a conserved set of molecular chaperones (Brehme *et al.*, 2014). Out of 219 molecular chaperone genes tested, knockdown of 18 led to increased paralysis rates in animals expressing A β in the muscle cells. The

majority of these genes are also protective in worms expressing polyglutamine, the aggregation of which is linked to Huntington's disease and a variety of other conditions, indicating that their effects may be related to a general improvement in protein homeostasis capacity (Brehme *et al.*, 2014). Another RNAi screen in the *C. elegans* model expressing A β in the muscle cells was targeted at genes with known human homologues, leading to the identification of various hits, including subunits of a vacuolar ATPase and of the ribosome, and a component of the TRiC/CCT chaperone (Khabirova *et al.*, 2014). Mitochondrial function was furthermore shown to be impaired in a model expressing A β 42 in the neurons (Fong *et al.*, 2016), and metabolomics and transcriptomics subsequently demonstrated that early changes in tricarboxylic acid (TCA) cycle metabolism were underlying this phenotype (Teo *et al.*, 2019).

The link between A β accumulation and the action of molecular chaperones was further supported by mass spectrometry, in a study where two Hsp70 proteins and three small heat shock proteins (sHSPs) were found to co-localise with A β deposits in the body wall muscle cells of *C. elegans* (Fonte *et al.*, 2002). In a study on an inducible *C. elegans* model expressing A β in body wall muscle cells, gene expression changes assessed by RNA microarrays also include sHSPs and Hsp70, as well as mitochondrial unfolded protein response (UPR) genes and proteasomal factors. An aggregation-prone GFP variant was employed as a control in this study, enabling the identification of the genes that are differentially expressed specifically as a consequence of A β -mediated toxicity, rather than resulting from protein aggregation in general (Hassan *et al.*, 2015). The changes in gene expression resemble those induced by a pore-forming toxin, Cry5B, suggesting that A β may exert its toxic effects in this model by affecting membrane integrity.

In a genome-wide screen in *C. elegans* expressing mutant tau in neurons, molecular chaperones have been identified along with proteases, kinases and phosphatases, and a number of other enzymes, signalling proteins and DNA/RNA regulating factors (Kraemer *et al.*, 2006). The identification of kinases and phosphatases is consistent with the known importance of tau phosphorylation in AD. However, RNAi knockdown of the *C. elegans* homologues of GSK3- β and MARK worsens the phenotype of the tau model, suggesting that the action of these kinases is beneficial, in contrast to their role in tau pathology in humans (Hanger, Anderton and Noble, 2009).

Fly models

Drosophila genetic screens are often based on screening for suppressors or enhancers of the rough eye phenotype caused by the expression of proteins that give rise to toxicity. Fly genetics have provided excellent tools for genome-wide studies, e.g. using randomly inserted transposable elements containing an

UAS (upstream activation sequence) that drives gene expression upon activation by the GAL4 protein, which can be expressed in a tissue-specific manner. Depending on the location and direction of insertion of the transposable element in the context of a gene, expression can either be enhanced or disrupted. Using this system, a screen has been performed on flies expressing A β 42 in the eye, leading to the identification of 23 modifier genes of which several have been confirmed by mutagenesis (Cao *et al.*, 2008). Consistent with the role of these pathways in human disease, genes operating in the secretory pathway and involved in cholesterol metabolism were found, as well as genes regulating chromatin structure and function (Cao *et al.*, 2008). In a complementary approach using a loss-of-function library, the gene *Toll* has been identified, which bears homology to the mammalian interleukin-1 receptor. Deletion of this gene or mutations in downstream signalling components ameliorate the rough eye phenotype and shorten the lifespan of the A β 42 flies, suggesting an important role of immune signalling in A β 42-mediated toxicity (Tan *et al.*, 2008).

A microarray transcriptomics study on flies expressing A β 42 throughout the nervous system revealed changes in pathways related to oxidative stress and the immune response (Favrin *et al.*, 2013). Although these pathways are also altered during the ageing of control flies, several genes are affected specifically by the expression of A β 42, such as the superoxide dismutase gene *Sod3* which is upregulated ~8-fold compared to controls. Further changes in the A β 42 flies include the upregulation of a gene involved in ER-to-Golgi transport, and the downregulation of a putative ER molecular chaperone, consistent with the involvement of the secretory pathway in A β 42 toxicity (Favrin *et al.*, 2013).

Drosophila tau models have yielded a set of pathways related to toxicity that are different from those in the A β models. Genetic screens using libraries of transposable element insertions led to the identification of phosphatases and kinases as modifiers of human tau toxicity in the fly eye (Shulman and Feany, 2003; Ambegaokar and Jackson, 2011), as well as diverse pathways including autophagy and lysosomal clearance, RNA processing, chromatin regulation, molecular chaperones and the cytoskeleton (Ambegaokar and Jackson, 2011). A RNAi screen on a tau R406W model also highlighted the role of the cytoskeleton, in particular dynein/dynactin mediated retrograde axonal transport, in addition to the activity of kinases and phosphatases, and protein metabolism (Butzlaff *et al.*, 2015). Although the role of kinases and phosphatases seems obvious, given the relationship between the phosphorylation state of tau and its propensity to aggregate and cause toxicity, the genetic interactions were shown to be rather complex (Ambegaokar and Jackson, 2011). The phosphorylation state of tau, as probed with the AT8 antibody, does not correlate with either enhancers or suppressors of aggregation. Furthermore, reduced expression of the GSK3- β homologue is a potent suppressor not only of tau toxicity but also of that induced by A β 42 and

TDP-43, suggesting that the network of kinases and phosphatases may play a role in common mechanisms underlying neurodegeneration (Ambegaokar and Jackson, 2011).

The *Drosophila* model based on tau V337M has been used to validate hits from GWAS performed on human AD patients. In one study, the relatively small number of participants did not produce statistically significant results, yet 6 out of 19 *Drosophila* homologues of candidate genes that were tested were shown to be modifiers of tau toxicity in the fly eye, including the glucose transporter *SLC2A14* (Shulman *et al.*, 2011). In a follow-up study of a larger number of GWAS-identified loci, 9 out of 84 genes were confirmed to be modifiers of toxicity including two genes involved in cell adhesion, *CD2AP* and *FERMT2* (Shulman *et al.*, 2014). Thus, this screening strategy appears to have a high success rate compared to that of unbiased screens, and can validate GWAS results below the threshold for statistical significance. It will be interesting to see whether or not *Drosophila* models based on A β 42 can confirm the roles of additional associated genes. A limitation is, however, presented by human genes which do not have *Drosophila* homologues, such as those involved in lipid metabolism (*APOE*, *CLU*) and immunity (*CRI*, *CD33* and *MS4A*) that are strongly associated with the risk of late-onset AD (Shulman *et al.*, 2014).

Rodent models

Genetic screening for modifiers of protein misfolding is not commonly undertaken in rodents, but analysis of gene expression levels and of protein abundance have been applied extensively to mouse models of AD to shed light on the cellular pathways that are disturbed in response to protein aggregation. Especially in recent years, methods of analysing gene expression using novel sequencing technologies, as well as methods in mass spectrometry to detect protein levels and modifications, have greatly expanded these possibilities.

A meta-analysis of the transcriptomes of aged and diseased humans versus mice has revealed that changes during ageing are relatively well-conserved between the two species, for example consistently showing upregulation of inflammatory factors (Hargis and Blalock, 2017). On the other hand, the gene expression profiles of mouse models of neurodegenerative diseases such as AD compared to healthy littermates display limited overlap with those of human patients (Burns *et al.*, 2015; Hargis and Blalock, 2017), showing, for example, less downregulation of genes associated with mitochondrial function, protein degradation, RNA processing and DNA repair (Burns *et al.*, 2015). Although the differences in gene expression between the animal models and human patients represent a major obstacle for the screening of drug candidates in preclinical trials, the models do allow perturbations in response to the misfolding of transgenically expressed proteins to be deciphered. Complementary insights into the dysregulation of cellular pathways

can be obtained from quantification of protein abundance, which does not always correlate directly with gene expression levels (Liu, Beyer and Aebersold, 2016), especially under conditions where protein quality control and turnover are perturbed. In the case of AD, transcriptomics and proteomics studies have together pointed towards a consistent set of dysregulated pathways in the various rodent models, including the immune response, mitochondrial function and synaptic transmission.

Activation of the immune response is generally thought to be a direct reaction to the deposition of A β plaques. In a comparative microarray analysis of brain tissue from Tg2576 and APPPS1 mice, the strongest change in gene expression was found to be a significant upregulation of the inflammatory factor progranulin, which was confirmed to be abundant in microglia and neurons in the vicinity of dense A β plaques (Pereson *et al.*, 2009). BRI-A β 42 mice similarly display progranulin immune-reactivity surrounding A β plaques, suggesting a direct effect of extracellular plaque formation in triggering this immune response (Pereson *et al.*, 2009).

Another study has attempted to separate events resulting directly from A β plaque formation from those caused by other sources of toxicity by comparing the gene expression pattern of two models, the 5xFAD model which has an aggressive phenotype with early onset A β plaque formation, and a model expressing an N-terminal A β truncated form (A β 4-42) that displays neuronal loss in the absence of A β plaques (Bouter *et al.*, 2014). Despite the different pathologies, both models suffer from age-related memory and learning defects of similar magnitudes. In that study, deep sequencing revealed that more than half of the differentially expressed genes in aged mice expressing A β 4-42 with respect to controls are similarly affected in the 5xFAD model, and that these are associated with in a broad range of biological functions including cell differentiation, neurogenesis, histone modification and transcription. Conversely, the majority of genes differentially expressed in the 5xFAD model compared to control mice are related to the immune response. These genes are not altered in the A β 4-42 mice, suggesting that these perturbations are a direct consequence of reactivity to A β plaques in the 5xFAD model. Astrocytes are, however, activated in both models, as indicated by the upregulation of *Gfap*, encoding the typical marker glial fibrillary acidic protein (Bouter *et al.*, 2014).

Microarray analysis of the gene expression profile of the 5xFAD mouse model also showed changes in genes involved in the immune response, pointing towards activation of the complement system and of interferon- γ regulated targets, concomitant with the appearance of pathological features including intracellular A β 42 accumulation, A β plaque deposition and cognitive symptoms (Landel *et al.*, 2014). A

mass spectrometric study specifically focusing on the activation of microglia has shown that levels of the cytokines IL1 β and IL10 are elevated before widespread plaque deposition occurs (Boza-Serrano *et al.*, 2018). A combined proteomics and genomics study on the 5xFAD mouse has also revealed changes in the expression of genes related to immune function, prior to the onset of A β plaque deposition (Bundy *et al.*, 2019). Other perturbations found in this study include the downregulation of proteins involved in the regulation of calcium binding, which show a trend in the same direction for mRNA and protein levels. 40% of the genes, however, were found to show an anticorrelation with their corresponding protein levels, underscoring the potential differences between gene expression levels and protein abundance, and the importance of complementing transcriptomics with proteomics.

Given the evidence for a role of mitochondrial dysregulation in AD, a detailed proteomic analysis of mitochondria isolated from 3xTg mice has been performed, leading to the identification of 23 proteins that are either up- or downregulated compared to non-transgenic mice (Chou *et al.*, 2011). These mitochondrial proteins have a variety of functions, including roles in metabolic processes such as oxidative phosphorylation but also functioning in ion transport, apoptosis and mitochondrial protein synthesis. The changes in protein levels are detectable in mice at 6 months of age, prior to widespread A β plaque deposition and tau tangle formation, thus reflecting a perturbation in mitochondrial homeostasis early in the disease process (Chou *et al.*, 2011). A similar approach on the APPPS1 mouse model has revealed changes in the levels of 60 mitochondrial proteins with roles in the electron transport chain, citric acid cycle, oxidative stress, and apoptosis, some of which are dysregulated prior to the onset of A β plaque formation (Völgyi *et al.*, 2017).

An integrated proteomics and transcriptomics study on another APP/PSEN1 model took the approach of first investigating changes at the proteome level in different brain regions, followed by validation of the corresponding genetic pathways at the mRNA level (Kempf *et al.*, 2016). Using mass spectrometry of peptides enriched in phosphorylation and glycosylation, changes in protein levels related to cytoskeleton-based synaptic plasticity were found, and the mRNA levels of upstream regulators such as glutamatergic receptors and neurotrophic factors were shown to be affected accordingly. Using quantitative mass spectrometry on APP, APP/PSEN1 and Bri-A β 42 mice, proteins associated with late-onset AD were found to be altered, most notably ApoE, which was seen to be upregulated in all three models (Savas *et al.*, 2017). Furthermore, proteins involved in excitatory synaptic transmission, in particular trafficking of the AMPA receptor, were found to be dysregulated, as well as mitochondrial proteins.

Several other reports have provided further evidence for the perturbation of synaptic plasticity at the proteome level. One study has specifically focused on the proteomic signature associated with memory defects, revealing an overlapping set of dysregulated membrane proteins involved in neuronal excitability and synaptic plasticity in the 5xFAD model of AD, as well as in aged control mice with memory impairment (Neuner *et al.*, 2017). Changes in the cytoskeleton as well as in synaptic function have been reported for the olfactory bulb of an APP/PSEN1 mouse model, occurring around the same age when A β plaques appear (Lachén-Montes *et al.*, 2016). Levels of cytoskeletal proteins as well as chaperones have in addition been suggested to be modulated by the presence of A β oligomers, as discovered in the APP Δ E693 mouse strain that accumulates oligomers rather than plaques (Takano *et al.*, 2013). Finally, the McGill mutant APP rat model displays changes in proteins related to stress response and synaptic function at a young age before the onset of A β plaques, whereas at later age metabolism (e.g. mitochondrial function and amino acid metabolism) becomes affected (Do Carmo *et al.*, 2018).

Analysis of the gene expression patterns of tau-based transgenic models has revealed important insights into the cellular responses towards tau aggregation. Transcriptional profiling of JNPL3 mice, which express tau P301L, revealed a down-regulation of anti-apoptotic factors, disturbances of microtubule-dependent intracellular trafficking, and down-regulation of a number of immune factors (Ho *et al.*, 2001). A study on another tau P301L model used more stringent selection criteria to identify just one upregulated gene, encoding glyoxalase I (Chen *et al.*, 2004). This enzyme functions in a pathway that prevents glycation and the formation of advanced glycated end products, which are known to occur in ageing and neurodegenerative disease. Glyoxalase I is strongly expressed in neurons with flame-shaped tau aggregates both in the mice and in AD patient brains, suggesting that it may be upregulated as a protective mechanism in response to protein aggregation (Chen *et al.*, 2004).

Changes in proteins functioning in metabolism, including mitochondrial proteins of the respiratory chain complex and detoxifiers of reactive oxygen species (ROS), as well as those with a role in synaptic transmission have furthermore been detected in a proteomic analysis of the tau P301L mouse model (David *et al.*, 2005). Injecting A β 42 fibrils into the amygdala of the tau P301L model has been found to result in further imbalances in components of the cytoskeleton, metabolism, and synaptic transmission, as well as proteins functioning in stress response pathways and protein folding and degradation (David *et al.*, 2006). Many of these changes have also been found in SHSY-5Y cells expressing tau P301L upon injection with A β 42 fibrils, suggesting conserved and rapid responses, whereas perturbations in the cytoskeleton and synaptic function appear exclusively in the mouse model, pointing towards longer-term or indirect consequences (David *et al.*, 2006). Proteomic analysis of postsynaptic density isolated from tau P301S mice

has shown a depletion of GTPase-regulatory proteins, which could be related to the actin cytoskeletal defects and loss of dendritic spines that are observed in this model (Dejanovic *et al.*, 2018). Consistent with many of these results, RNAseq of a more recent tau P301S mouse model has furthermore revealed perturbations in genes associated with presynaptic factors, glutamatergic signalling, and protein scaffolding (Ke *et al.*, 2019).

An additive effect of A β and tau toxicity on mitochondrial dysfunction has been found in a triple mouse model, combining familial APP, PSEN2 and tau mutations (Rhein *et al.*, 2009). Mitochondrial proteins including Complex I and Complex IV subunits are downregulated at the protein level in the triple mouse model more strongly than in the parental APP/PSEN2 or tau strains. In support of these findings, the respiratory function of isolated mitochondria was found to be reduced more drastically and at earlier age in the triple model compared to the APP/PSEN2 strain or wild-type animals (Rhein *et al.*, 2009).

Proteomic approaches have moreover been used to assess the misfolding and aggregation of endogenous mouse proteins, which may be triggered by the pressure on the protein homeostasis machinery caused by the aggregation of transgenic A β and tau. Analysis of the SDS-insoluble fraction from mice expressing APP with Swedish mutations K670N/M671L and PSEN1 Δ exon9 has shown the presence of cytosolic proteins including glycolytic enzymes and members of the 14-3-3 family of chaperones (Xu *et al.*, 2013). In an independent study on the same mouse model using quantitative proteomics, mitochondrial proteins have been identified in the sarkosyl-insoluble fraction of aged mice (Thygesen *et al.*, 2018). Finally, recent work on the inducible tau model rTg4510 has led to a set of SDS-insoluble proteins including kinases, other enzymes and chaperones that partially overlaps with that of the APP/PSEN1 model established using the same methods (Pace *et al.*, 2018), suggesting a common mechanism that triggers the misfolding of otherwise soluble proteins.

4.2 Biological pathways in Parkinson's disease models

Yeast models

The genetic toolkit available for screens in yeast has been applied extensively to α -synuclein models, leading to the discovery of conserved cellular pathways involved in its toxicity. Early screens have made use of a genome-wide deletion library, showing an enrichment for genes involved in lipid metabolism and vesicle-mediated transport (Willingham *et al.*, 2003). In accordance with the latter, a genome-wide

overexpression screen for suppressors and enhancers of α -synuclein toxicity identified the yeast homologue of human Rab1, a GTPase involved in ER-to-Golgi vesicle trafficking, as an important suppressor of toxicity. This protein is often found to co-localise with α -synuclein cytoplasmic inclusions, and its overexpression restores the trafficking defects associated with α -synuclein toxicity (Cooper *et al.*, 2006). Yeast homologues of Rab3a and Rab8a, which function in synaptic and post-Golgi vesicle transport, respectively, have also been found associated with the inclusions in subsequent work, and overexpression of any one of these three Rab proteins rescues toxicity in *C. elegans* expressing α -synuclein in dopaminergic neurons (Gitler *et al.*, 2008).

In addition to defects in ER-to-Golgi trafficking, mitochondrial dysfunction has been identified in a transcriptional screen of a yeast strain with high α -synuclein expression levels, together with an increase in reactive oxygen species (ROS) (Su *et al.*, 2010). A library screen on this model has revealed a group of 1,2,3,4-tetrahydroquinolinones capable of reducing α -synuclein foci formation and toxicity, in addition to re-establishing ER-to-Golgi trafficking as well as mitochondrial function, suggesting that these processes are tightly linked. These compounds are furthermore active in a *C. elegans* model and in rat primary neuron cultures, providing evidence for a conserved mechanism (Su *et al.*, 2010).

The role of vesicle trafficking has been confirmed once more in a study that made use of the high sensitivity to hydrogen peroxide of the yeast strain expressing α -synuclein (Liang *et al.*, 2008). This study in addition reported ubiquitin-dependent protein degradation, protein biosynthesis, and stress response genes as suppressors of toxicity. The *YPP1* gene was discovered as a suppressor specifically for the α -synuclein A30P mutant, and the encoded protein was shown to target α -synuclein to vacuoles for degradation (Flower *et al.*, 2007). Findings of another overexpression screen using a large library of 5,500 strains, covering 85% of the yeast genome, are largely in agreement with the pathways previously mentioned, in addition pointing to a role for protein phosphorylation in the toxicity of α -synuclein (Yeager-Lotem *et al.*, 2009). In this screen, the yeast homologue of human *PARK9* was also identified, which encodes a lysosomal P-type ATPase that is mutated in a familial form of PD (Gitler *et al.*, 2009). Overexpression of this gene suppresses α -synuclein toxicity not only in yeast, but also in a *C. elegans* model and in rat primary dopaminergic neurons. It also protects yeast cells against manganese-induced toxicity, a known risk factor for PD, providing a potential link between genetic and environmental factors in initiating the disease process (Gitler *et al.*, 2009). Two other hits from this screen, an E3 ubiquitin ligase and a factor related to nitrosative stress, have later been validated in cortical neurons differentiated from patient-derived induced pluripotent stem cells (iPSCs) (C. Y. Chung *et al.*, 2013).

Building on the results from genetic screens, a computational approach was designed with the aim of creating a genetic network underlying the disease phenotype, and of transposing it to the human homologues. Thus, a genetic map of α -synuclein toxicity has been created which spans the pathways identified previously, ranging from ER quality control and vesicle trafficking to calcium signalling and mitochondrial function, in which known genetic causes of familial PD appear in different parts of the network (Khurana *et al.*, 2017). In an accompanying study that employs *in situ* peroxidase labelling coupled to mass spectrometry, the physical interaction partners of α -synuclein in rat primary cortical neurons were found to form a proteomic network that largely overlaps with the genetic network (Chung *et al.*, 2017). Altogether, the yeast α -synuclein models in combination with available genomic and proteomic tools have generated remarkable results in understanding the processes underlying PD.

Worm models

In agreement with the findings in yeast, several independent genetic screens in *C. elegans* found components of vesicle trafficking pathways to be involved in α -synuclein accumulation and toxicity. A genome-wide RNAi screen for suppressors of inclusion formation in the *C. elegans* model expressing α -synuclein in the body wall muscle cells revealed an enrichment of genes associated with the ER/Golgi and vesicle transport (van Ham *et al.*, 2008). In a study targeting a subset of genes known or hypothesised to be involved in PD, two vesicle trafficking genes were discovered that protect against α -synuclein-mediated degeneration of dopaminergic neurons, in addition to known genetic PD factors such as *DJ-1* and *PINK1* (Hamamichi *et al.*, 2008). Finally, another screen performed on a pan-neuronal α -synuclein model led to the detection of several genes involved in endocytosis that protected against α -synuclein neurotoxicity, including two subunits of the adapter protein complex AP2 (Kuwahara *et al.*, 2008).

A gene that seemingly operates in a separate pathway that promotes aggregation was originally discovered in a screen targeting polyglutamine aggregation in the body wall muscle cells of *C. elegans*. This gene, *moag-4* or *SERF* in humans, is similarly active in driving α -synuclein inclusion formation and toxicity, independently of known chaperone-dependent or insulin-signalling pathways (van Ham *et al.*, 2010). Although the function of *SERF* in humans is not known at present, this gene may represent a suitable target for therapeutic intervention in protein misfolding diseases.

Fly models

Drosophila models of α -synuclein toxicity have been used less extensively in the context of unbiased genetic screens. Using the reduced levels of dopamine in the heads of flies expressing α -synuclein A53T as a read-out, in combination with a genome-wide deletion library, the mitochondrial chaperone TRAP1 has been identified as a potent suppressor of toxicity (Butler *et al.*, 2012). Overexpressing TRAP1 in the α -synuclein A53T flies partially restores their climbing ability and resistance to oxidative stress, and the latter has been confirmed in rat neurons as well as in human cell lines, pointing towards a conserved link between α -synuclein pathology and mitochondrial dysfunction that is thought to play an important role in PD (Vicario *et al.*, 2018).

Rodent models

Several transcriptomics and proteomics studies have been performed in recent years in rodent models for PD based on the transgenic expression of α -synuclein. One investigation was carried out on a cross of an A53T α -synuclein overexpression model and a knock-out strain of the mitochondrial kinase PINK1, which causes an enhanced phenotype with ubiquitinated α -synuclein inclusions in the spinal cord and basal brain in mice from one year of age, and a reduced lifespan (Gispert *et al.*, 2015). Transcriptomic analysis of brain samples taken before the appearance of α -synuclein inclusions in this model has revealed perturbations in genes related to mitochondrial function, ubiquitin-mediated degradation, microtubule and vesicle trafficking, synaptic signalling and DNA-damage repair, compared to wild-type animals. At the protein level, analysis of ubiquitination patterns in 18-month-old mice has provided evidence for enhanced ubiquitination of α -synuclein itself upon the loss of PINK1, as well as reduced ubiquitination of the histone variant H2AX, a marker of sites of DNA-damage (Gispert *et al.*, 2015).

A quantitative proteomics study on the model expressing α -synuclein with both A30P and A53T mutations has also highlighted the role of mitochondrial dysfunction and oxidative stress in the substantia nigra, as well as impairment of the ubiquitin-proteasome system, and endoplasmic reticulum (ER) stress (Yan *et al.*, 2017). A useful data source was furthermore established by a detailed analysis of the protein levels in distinct regions of the mouse brain, which has been performed for both non-transgenic mice and the Line 61 PD mouse model (S. Y. Jung *et al.*, 2017). Comparison of the substantia nigra and an unaffected brain region in the PD model showed that membrane signalling proteins are upregulated specifically in the substantia nigra, whereas markers of extracellular matrix remodelling and inflammation are increased in aged PD mice compared to non-transgenic mice.

Further studies at the systems level have mostly been performed on rodent models of PD induced by

treatment with neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which have been remarkably successful in recapitulating phenotypic aspects of human PD (Tieu, 2011; Kasahara *et al.*, 2017). Although these studies have identified perturbations in common pathways including oxidative phosphorylation, oxidative stress, apoptosis and synaptic transmission (Smith, 2009), a comparative meta-analysis revealed that the gene expression signature of these models is relatively remote from that of human PD patients (Oerton and Bender, 2017). Overall, current animal models of PD provide useful tools to study certain aspects of the multi-factorial disease process, such as the interplay between mitochondrial dysfunction and α -synuclein aggregation, but need to be improved to recapitulate better the complexity of the disease in the human brain.

5. Methodological challenges

An essential aim of *in vivo* models of human disease is to enable preclinical studies on systems that recapitulate the pathological processes observed in patients. In this section we discuss four major challenges, two biophysical and two biological, that remain to be addressed in order to reach this goal:

(1) To develop longitudinal detection methods for protein aggregates. In AD and PD drug discovery pipelines, preclinical studies in mouse models often rely on the post-mortem quantification of protein deposits using methods based on immunohistochemistry or amyloid-sensitive fluorescent dyes (Sevigny *et al.*, 2016; Drummond and Wisniewski, 2017). Methods based on clearing techniques, including CLARITY (Chung and Deisseroth, 2013; K. Chung *et al.*, 2013), 3DISCO (Ertürk *et al.*, 2012) and Scale (Hama *et al.*, 2011) (**Table 1**), enable more quantitative assessments of the total amounts and distributions of these deposits, thereby offering more accurate endpoints on the anti-aggregation activities of therapeutic candidate compounds.

One may expect even better readouts, however, by the further development of methods of monitoring longitudinally the inhibitory effects of these compounds. Approaches based on magnetic resonance imaging (MRI) and positron emission tomography (PET) (**Table 1**) (Weiner *et al.*, 2013; Sevigny *et al.*, 2016) offer already the possibility of carrying out this type of studies in mouse models. The development of PET tracers for α -synuclein will be particularly helpful in this direction (Merchant *et al.*, 2019).

(2) To develop detection methods for protein oligomers. Both in AD and PD the most neurotoxic species are misfolded protein oligomers, and yet these species remain elusive and difficult to quantify, in

particular in *in vivo* models (**Table 1**). Immunohistochemistry is typically used to probe the accumulation of A β , tau and α -synuclein in fixed samples, and conformation-specific antibodies are available for specific cases, including A11, which binds to β -sheet oligomers, and MC-1, which recognises misfolded tau. The recent introduction of ELISA-based methods of greatly enhanced sensitivity, such as SIMOA (Kuhle *et al.*, 2016), subject to further development, is opening novel opportunities in this direction. In our laboratory we have recently demonstrated the design of antibodies targeting specific epitopes (Sormanni, Aprile and Vendruscolo, 2015; Aprile *et al.*, 2017)[Aprile *et al.* PNAS 2020 in press], and this approach holds promise for discriminating different populations of oligomeric species based on the accessibility of a range of epitopes.

Advances in microscopy are furthermore expected to contribute to an improved molecular understanding of misfolded protein species *in vivo*. Super-resolution microscopy has revealed the morphology and the growth of fibrils at the nanometer scale *in vitro* and in cells (Kaminski Schierle, Van De Linde, *et al.*, 2011; Duim *et al.*, 2014; Pinotsi *et al.*, 2016), and continuing efforts to implement these technologies *in vivo* have already allowed the use of RESOLFT and STED nanoscopy to visualise organelles and the cytoskeleton in *C. elegans* (Dreier *et al.*, 2019), *Drosophila* (Schnorrenberg *et al.*, 2016), and the mouse brain (Berning *et al.*, 2012). Furthermore, the use of biosensors in combination with fluorescence microscopy may shed more light on the conformational state of protein aggregates *in vivo*, as demonstrated e.g. by the changes in the fluorescence lifetime of protein tags upon amyloid formation (Kaminski Schierle, Bertonecini, *et al.*, 2011; Laine *et al.*, 2019).

Another approach that holds promise for the detection of oligomeric protein species in animal models and patient material relies on seeded amplification *in vitro*. Several assays, e.g. protein misfolding cyclic amplification (PMCA) (Saborio, Permanne and Soto, 2001), the amyloid seeding assay (Colby *et al.*, 2007) and real-time quaking-induced conversion (RT-QuIC) (Atarashi *et al.*, 2011) (**Table 1**), were originally designed to detect low concentrations of misfolded prion protein molecules, based on the conversion of PrP^C to PrP^{Sc} by the addition of seeds of the latter species. These assays have been adapted successfully to enable the detection of misfolded α -synuclein (B. C. Jung *et al.*, 2017; Shahnawaz *et al.*, 2017) and tau (Meyer *et al.*, 2014) *in vitro*, from mice and from human brain or cerebrospinal fluid (CSF). Further development of these approaches will undoubtedly help not only with the fundamental understanding of protein misfolding *in vivo*, but may also lead to diagnostic methods for patients.

(3) To create models to recapitulate the sporadic forms of AD and PD. The overexpression of A β , tau and α -synuclein or their mutational variants provides models that recapitulate the familial forms of AD and

PD. In the sporadic forms, more subtle perturbations of the protein homeostasis system are probably at play, which result in the aggregation of the disease-associated proteins starting from normal expression levels. The creation of specific animal models is becoming technically more feasible with developments in genetic engineering techniques such as CRISPR/Cas9 (Knott and Doudna, 2018; Zhang, 2019), and by introducing additional genetic or environmental perturbations the need for the artificially high overexpression levels and non-physiological mutations on which current models often rely may be alleviated. For example, we have recently created a *C. elegans* model expressing A β 42 at single-copy levels in only two neurons, which displays small perturbations in neuronal signalling and may be useful to study the onset of disease symptoms triggered by additional sources of cellular stress (Sinnige *et al.*, 2019).

(4) To create models to recapitulate the complexity of human AD and PD brain tissues. The *in vivo* models that we have described in this review have the inevitable limitation that they are genetically non-identical to humans, leading to differences in neuronal anatomy as well as processes such as ageing and the action of the immune system, which are critical in the study of neurodegenerative diseases. The use of iPSC-derived neurons and direct reprogramming of patient fibroblasts are promising approaches to circumventing some of these issues (Xu, Du and Deng, 2015; Engle, Blaha and Kleiman, 2018). In combination with 2D and 3D culturing methods, such as neuronal rosettes and brain organoids, respectively (Choi *et al.*, 2014; Otani *et al.*, 2016; Lancaster *et al.*, 2017; Gonzalez *et al.*, 2018), these systems may increasingly provide avenues to elucidate disease mechanisms and to screen for therapeutics, in parallel to the use of traditional model organisms.

6. Conclusions

The use of *in vivo* models is instrumental in obtaining a detailed understanding of human diseases by connecting the observed phenotypes to the underlying molecular processes that give rise to them. In this review we have described current *in vivo* models of Alzheimer's and Parkinson's diseases where the phenomenon of protein misfolding and aggregation plays a central role.

Studies based on these models have shown that mature amyloid fibrils or large aggregate inclusions are generally relatively inert and not associated with cytotoxicity, as they are limited in their ability to engage in aberrant interactions. By contrast, aggregation intermediates such as oligomeric species formed by A β , tau or α -synuclein are associated with the disturbance of cellular processes and with the onset and progress of neurodegenerative diseases. Some of the affected pathways appear to be disease-specific, as exemplified

by the association of vesicle trafficking and mitochondrial function with the misfolding of α -synuclein, and of cytoskeletal transport and protein phosphorylation with that of tau. Importantly, protein homeostasis becomes progressively impaired in ageing and neurodegeneration, and molecular chaperones have been shown to interact with all the three proteins discussed in this article, and to modulate their misfolding and aggregation.

In perspective, however, the pathological mechanisms underlying these disorders are not yet fully understood, and no disease-modifying therapeutics have yet emerged. The creation of *in vivo* models that better recapitulate the sporadic forms of these diseases, as well as of accurate biophysical methods for the accurate quantification of the aggregation process in living organisms, will be crucial in this regard.

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Fig. 1. Mechanisms of amyloid formation *in vitro*. (a) *In vitro* ThT aggregation assay of A β 42 at different starting monomer concentrations. The curves are fitted using a model that includes both primary and secondary nucleation. Figure adapted from (S. I. A. Cohen *et al.*, 2013). (b) Schematic of the kinetic model of fibril formation including primary and secondary processes. Soluble monomeric forms of proteins undergo primary nucleation to generate oligomeric species that have the potential to convert into fibrils. The formation of fibrils is significantly enhanced by secondary processes that enable aggregates to proliferate by the further association of soluble protein molecules. The latter processes include fragmentation, which generates new fibril ends at which growth occurs, and surface catalysed nucleation, in which fibril surface functions as a template for the generation of new oligomeric and fibrillar species. Figure based on (S. I. A. Cohen *et al.*, 2013). (c) ThT aggregation assay of α -synuclein in the presence of different concentrations of dimyristoylphosphatidylserine (DMPS) vesicles (black, 60 μ M; purple, 120 μ M; dark blue, 180 μ M; light blue, 240 μ M; dark green, 300 μ M; light green, 450 μ M; yellow, 600 μ M; orange, 1200 μ M). Figure adapted from (Galvagnion *et al.*, 2015). (d) Schematic of the dominating mechanism of amyloid formation by α -synuclein in the presence of small unilamellar lipid vesicles, which present an interface for nucleation to occur. Figure based on (Galvagnion *et al.*, 2015).

Fig. 2. Schematic of A β , tau and α -synuclein, and of the mutations that have been used in the models described in this article. (a) APP and its sequential processing into the A β peptide by β -secretase and γ -secretase. Cleavage sites are indicated by the residue number at the N-terminal side of the cleavage; mutations are indicated with the residue numbers of full-length APP and those of the A β 40/42 cleavage product. Other APP processing pathways, such as α -secretase processing, and other A β forms, such as A β 39 or A β 43, are omitted for clarity. (b) The *MAPT* gene encoding tau undergoes splicing to yield six possible isoforms. Shown is the full-length 2N4R tau isoform of 441 residues; 1N and 0N isoforms lack the second or both N-terminal inserts, respectively, and 3R isoforms lack the second microtubule repeat (dashed boxes). The hexapeptide stretches VQIINK and VQIVYK, which have the propensity to form β -strands and are essential for the aggregation of tau, are located at the microtubule binding repeats. (c) Schematic of α -synuclein, containing three major regions: an N-terminal amphipathic domain with α -helical propensity

where several familial mutations are located (residues 1–61), a central hydrophobic part (residues 61–95) known as the NAC region that is essential for amyloid formation, and an acidic C-terminal region (residues 95–140).

Fig. 3. Examples of *in vivo* models of protein misfolding for the study of Alzheimer's and Parkinson's diseases. (a) The budding yeast *S. cerevisiae* is the most widely used eukaryotic model organism, featuring a conserved cellular organisation, including the nucleus and the secretory system. (b) The expression of two copies of human α -synuclein (either in the wild-type or the familial A53T and A30P forms) in *S. cerevisiae* leads to the formation of cytoplasmic puncta for the wild-type and A53T variants, but not for A30P; reproduced from (Outeiro and Lindquist, 2003) with permission from Science. (c) The nematode worm *C. elegans* is a multicellular organism that consists of several tissues, such as a digestive system comprised of the pharynx and the intestine. (d) Left: *C. elegans* expressing A β 42 in the body wall muscle cells display deposits that can be stained with the amyloid-binding dye X-34. The head region of an A β 42-expressing animal is shown, with deposits indicated by arrows; asterisks mark unspecific staining of the mouth and intestine. Right: close-up of *C. elegans* expressing A β 42 in the two BAG neurons, stained with an anti-A β antibody (green) and DAPI (blue). Adapted from (Sinnige *et al.*, 2019). (e) The fruit fly *D. melanogaster* has a more complex brain than *C. elegans*, and possesses a compound eye that is often used as a read-out for toxicity. (f) The expression of A β 42, but not of A β 40, leads to the formation of ThS-positive deposits. Arrows indicate deposits; Kn: Kenyon cell layer; Ca: calyx. Reproduced from (Iijima *et al.*, 2004); copyright 2004 National Academy of Sciences. (g) Rodent models are typically used for pre-clinical studies, but also provide a more complex system for fundamental studies of human disease mechanisms. (h) Overexpression in mice of human APP and tau with familial mutations leads to the formation of A β deposits positive for ThS staining (left) and structures resembling tau tangles as visualised by Bielschowsky silver staining (right). The arrow in the left panel indicates a neuron. Reproduced from (Ribé *et al.*, 2005) with permission from Elsevier.

Table 1: Commonly used biophysical methods for the study of protein misfolding *in vivo* and *in vitro*. The application of these methods to study protein aggregation and the associated references are mentioned throughout the main text.

Methods and tools to probe protein misfolding and aggregation <i>in vivo</i> and <i>in situ</i>	
<i>Amyloid binding dyes</i>	ThS, Congo red, NIAD-4, X-34, K114, luminescent conjugated oligothiophenes (LCOs), silver stain (tau tangles)
<i>Microscopy</i>	EM, immunohistochemistry, fluorescence microscopy including FLIM and FRAP
<i>Conformation-specific antibodies</i>	A11 (oligomers), MC1 (misfolded tau), TOC1 (tau oligomers), FILA-1 (α -synuclein oligomers), DesAbs (designed antibodies)
<i>Detergent solubility</i>	Sarkosyl, SDS
<i>Size determination</i>	SEC, sucrose gradient, SDS PAGE, western blot
<i>Visualisation of deposits</i>	CLARITY, 3DISCO, Scale, MRI, PET
<i>Biochemical assays</i>	ELISA, SIMOA , amyloid seeding assay, PMCA, RT-QuIC
Methods to probe cellular pathways <i>in vivo</i> and <i>in situ</i>	
<i>Genetic screens</i>	RNAi, random mutagenesis
<i>Transcriptomics</i>	RNA microarrays, next generation sequencing (RNAseq)
<i>Proteomics</i>	TMT, iTRAQ, label-free mass spectrometry
Methods to probe protein aggregation <i>in vitro</i>	
<i>Aggregation kinetics</i>	ThT fluorescence, quartz crystal microbalance, filter trap assay
<i>Secondary structure determination</i>	CD spectroscopy, FTIR spectroscopy
<i>Size/shape determination</i>	DLS, SEC-MALS, smFRET, SAXS, AFM, super-resolution microscopy
<i>3D structure determination</i>	Cryo-EM, solid-state NMR spectroscopy, X-ray diffraction

Table 2: List of yeast models of protein misfolding in Alzheimer's and Parkinson's diseases.

Misfolded protein	Construct expressed	Organismal phenotype	Protein aggregation behaviour	Pathways (proteins) involved	Primary reference
<i>Alzheimer's disease</i>					
A β	A β 42-Sup35p MRF	not determined	SDS-stable oligomers	heat shock response (Hsp104)	(Bagriantsev and Liebman, 2006)
	A β 42-GFP and GFP-A β 42	reduced growth rate	punctate patches; co-localisation with lipids	heat shock response	(Caine <i>et al.</i> , 2007)
	A β 42 with an ER targeting sequence	reduced growth rate	oligomers not resistant to boiling or SDS	endocytosis (yeast PICALM)	(Treusch <i>et al.</i> , 2011)
	A β 42 with an ER targeting sequence	reduced growth rate	Oligomers	respiration, proteasomal degradation	(Chen and Petranovic, 2015)
	MF α -A β 42-GFP WT and E22G	reduced viability and growth rate	accumulation in the secretory pathway	membrane trafficking (yeast PICALM)	(D'Angelo <i>et al.</i> , 2013)
tau	tau 2N3R and 2N4R	no change in growth rate	hyperphosphorylated and misfolded tau; small sarkosyl-insoluble fraction	phosphorylation (yeast GSK-3 β and CDK5)	(Vandebroek <i>et al.</i> , 2005)
	tau 2N4R WT, P301L, G272V, N279K, Δ K280, V337M, R406W, S409E, S409A	no change in growth rate	phosphorylation of S409 increases sarkosyl-insoluble aggregates and higher order oligomers	oxidative stress; mitochondrial dysfunction	(Vanhelmont <i>et al.</i> , 2010)
<i>Parkinson's disease</i>					
α -synuclein (α syn)	α syn-GFP WT, A30P, A53T	reduced growth rate at higher expression levels; less severe for A30P	inclusions for WT and A53T co-localising with ubiquitin; A30P diffusely distributed	proteasomal degradation; lipid metabolism; ER-to-Golgi vesicle trafficking	(Outeiro and Lindquist, 2003)
	α syn-GFP WT, A30P, A53T	no reduction in growth rate or viability	WT and A53T form inclusions at the plasma membrane; A53T inclusions are ThS positive; A30P diffuse	lipid synthesis; proteasome; autophagy; endocytosis; oxidative stress	(Zabrocki <i>et al.</i> , 2005)
	GFP/GST- α syn WT, A30P, A53T	reduced viability for overexpression of WT and A53T but not A30P	inclusions for WT and A53T but not for A30P; GST-fusions of WT and A30P form soluble oligomers	heat shock response; proteasomal degradation	(Dixon <i>et al.</i> , 2005)
	GFP- α syn WT, A30P, A53T	reduced growth rate for all 3 strains	inclusions for WT and A53T but not for A30P	heat shock response; oxidative stress; apoptosis	(Flower <i>et al.</i> , 2005)

Table 3: List of worm models of protein misfolding in Alzheimer's and Parkinson's diseases.

Misfolded protein	Protein expressed	Tissue	Organismal/cellular phenotype	Protein aggregation behaviour	Pathways (genes) involved	Primary reference
<i>Alzheimer's disease</i>						
A β	A β 3-42 with signal peptide	body wall muscle cells	paralysis phenotype	ThT and X-34 positive deposits, fibrils in EM	molecular chaperones (ER Hsp70, sHSPs)	(Link, 1995)
	A β 1-42 with signal peptide	body wall muscle cells	paralysis phenotype	ThT and X-34 positive deposits	molecular chaperones (Hsp70, Hsp90, TRiC/CCT and others); lysosomal pathways (subunit of vacuolar ATPase); protein synthesis (ribosomal subunits)	(McColl <i>et al.</i> , 2012)
	A β 3-42 with signal peptide	neurons	chemotaxis and learning impairments	oligomeric species recognised by NU-4 antibody		(Wu <i>et al.</i> , 2006)
	A β 1-42 with signal peptide	neurons	neuromuscular defects, reduced food sensing	insoluble protein observed at old age	metabolism, mitochondrial function	(Fong <i>et al.</i> , 2016)
	A β 42 with signal peptide	glutamatergic neurons	neurodegeneration of the tail glutamatergic neurons	ND		(Treusch <i>et al.</i> , 2011)
	A β 1-42 with signal peptide	BAG neurons	subtle modulation of behavioural response to CO ₂	no ThT or X-34 staining observed	calcium signalling	(Sinnige <i>et al.</i> , 2019)
tau	tau 1N4R WT, P301L and V337M	neurons	degeneration of axons, uncoordinated phenotype (stronger for mutants)	phosphorylation, accumulation of insoluble tau (more/faster for mutants)	protein phosphorylation; chaperones; proteasomal degradation	(Kraemer <i>et al.</i> , 2003)
	tau 3R and 4R WT, 4R P301L and 4R R406W	mechanosensory neurons	WT slightly impaired touch response; mutants strong age-related decline in touch response; neurodegeneration; loss of microtubules	accumulation in cell bodies and neurites; no signs of aggregation	kinases (GSK3); chaperones (Hsp70)	(Miyasaka <i>et al.</i> , 2005)
	tau 3R WT, PHP (phosphomimic)	neurons	partial uncoordinated phenotype and reduced thrashing; PHP more gaps in axons	protein in detergent-soluble but not insoluble fraction		(Brandt <i>et al.</i> , 2009)
	tau 1N4R V337 + tau F3 Δ 280 fragment	neurons	worsened locomotory phenotype and neuronal abnormalities compared to tau V337M alone; perturbed mitochondrial transport	ThS positive inclusions		(Fatouros <i>et al.</i> , 2012)

amyloid- β + tau	A β 42 + tau F3 Δ 280 fragment	neurons	shortened lifespan and worse defects in egg laying and chemotaxis compared to single transgenic lines; dopaminergic neurodegeneration	more Congo Red positive deposits compared to single transgenic lines	protein phosphorylation; ageing	(Wang <i>et al.</i> , 2018)
<i>Parkinson's disease</i>						
α -synuclein (α syn)	α syn-YFP	body wall muscle cells	motility defect	cytoplasmic inclusions, largely mobile until old age	vesicle trafficking; lipid metabolism	(van Ham <i>et al.</i> , 2008)
	α syn-GFP	body wall muscle cells	not determined	cytoplasmic inclusions	PD related pathways (pink, parkin); endolysosomal pathways; autophagy	(Hamamichi <i>et al.</i> , 2008)
	α syn WT, A53T	neurons	dopaminergic neuronal loss; motor deficit	rare inclusions		(Lakso <i>et al.</i> , 2003)
	α syn WT	dopaminergic neurons	neurodegeneration	no inclusions observed	molecular chaperones (torsinA)	(Cao <i>et al.</i> , 2005)
	α syn WT, A53T, A30P	dopaminergic neurons	loss of dendrites and behavioural defects in A53T and A30P	occasional clump/dot like distribution		(Kuwahara <i>et al.</i> , 2006)
	α syn WT, A53T, A30P	neuronal	no motility or development phenotype	not determined	vesicle trafficking	(Kuwahara <i>et al.</i> , 2008)
	α syn WT-YFP, designed proline mutants	muscle cells	not determined	WT forms elongated structures; triple proline mutant remains diffuse		(Karpinar <i>et al.</i> , 2009)

Table 4: List of fruit fly models of protein misfolding in Alzheimer's and Parkinson's diseases.

Misfolded protein	Construct expressed	Tissue	Organismal/cellular phenotype	Protein aggregation behaviour	Pathways (genes) involved	Primary reference
<i>Alzheimer's disease</i>						
A β	APP + β -secretase	eye	neurodegeneration of retina and neuropil of the optical lobe	ThS positive deposits in retina and neuropil with a star-like appearance in EM		(Greeve <i>et al.</i> , 2004)
	APP WT, K670N/M671L + β -secretase	neurons	age-associated memory defects and neurodegeneration	SDS stable A β oligomers; A β deposition in cortical layer		(Sarantseva <i>et al.</i> , 2009)
	APP + β -secretase	neurons	impaired climbing and memory; age-related neurodegeneration; developmental defects when raised at higher temperature	A β deposition; punctate X-34 positive deposits when raised at higher temperature		(Chakraborty <i>et al.</i> , 2011; Mhatre <i>et al.</i> , 2014)
	A β 42*	eye, neurons	rough eye phenotype; locomotory and learning defects; shortened lifespan; neurodegeneration	ThS positive deposits; A β mostly insoluble	secretory pathways; cholesterol homeostasis; regulation of chromatin structure and function; immune response (Toll)	(Finelli <i>et al.</i> , 2004; Iijima <i>et al.</i> , 2004)
	A β 42 WT and E22G	neurons	rough eye phenotype; neurodegeneration; locomotor defects; shortened lifespan	intracellular A β accumulation; diffuse extracellular deposits positive for oligomer antibody but not for Congo red; E22G forms SDS-resistant tetramers		(Crowther <i>et al.</i> , 2005)
	16 variants of A β 42	neurons	locomotor defects and shortened lifespan	not determined experimentally; good correlation between severity of phenotype and predicted aggregation propensity		(Luheshi <i>et al.</i> , 2007)
	tandem A β 40, tandem A β 42	eye, neurons	tandem A β 42 causes rough eye phenotype and reduced lifespan; tandem A β 40 no effect	both form SDS-insoluble aggregates; tandem A β 42 forms soluble oligomeric species		(Speretta <i>et al.</i> , 2012)
	A β 42 WT, E22G, L17P	neurons	learning and locomotory defects; shortened lifespan	intracellular ThS positive deposits; oligomers for WT and E22G		(Iijima and Iijima-ando, 2008)
	A β 42 WT, E22G	giant fiber neurons	locomotory defects; shortened lifespan; failed synaptic transmission; depletion of presynaptic mitochondria and synaptic vesicles	accumulation in the cell bodies and axons	synaptic transmission	(Zhao <i>et al.</i> , 2010)
tau	tau WT, R406W	(cholinergic) neurons	shortened lifespan; age-related neurodegeneration	misfolded and phosphorylated tau; no fibrils found	protein phosphorylation; cell adhesion	(Wittmann <i>et al.</i> , 2001)

	tau 2N4R WT + GSK3- β	eye	neurodegeneration starting from larval stage	flame shaped aggregates; PHFs and SFs in EM; tangles positive for phosphorylated tau	autophagy and lysosomal pathways; RNA processing; chromatin regulation; molecular chaperones; cytoskeleton	(Jackson <i>et al.</i> , 2002)
	tau 0N4R WT	glia	reduced lifespan; increased apoptosis	glial inclusions of phosphorylated, ThS and Bielschowsky silver positive tau; fibrils in sarkosyl-insoluble fractions in aged flies		(Colodner and Feany, 2010)
	tau 0N4R WT, R5L, S320F, S352L, G389R, R406W	neurons, glia	various types of cellular stress	inclusions of phosphorylated tau	apoptosis; oxidative stress (JNK signalling); autophagy; unfolded protein response	(Bardai <i>et al.</i> , 2018)
amyloid- β + tau	A β 42 + tau 1N4R WT, R406W	(motor) neurons	exacerbated abnormal axon morphology, locomotory defects and shortened lifespan compared to single transgenics	stronger tau phosphorylation; aggregation not examined		(Folwell <i>et al.</i> , 2010)
<i>Parkinson's disease</i>						
α -synuclein (α syn)	α syn WT, A30P, A53T	neurons	Loss of dopaminergic neurons; climbing impairment	Lewy body like round inclusions with radiating fibrils, thread and grain-like structures (EM)		(Feany and Bender, 2000)
	α syn WT, A30P, A53T	dopaminergic neurons	loss of dopaminergic neurons	Lewy body-like and Lewy neurite-like staining; α syn mostly soluble in high salt/ detergent buffer	heat shock response (Hsp70)	(Auluck <i>et al.</i> , 2002)
	α syn WT	neurons	widespread neurodegeneration; reduced lifespan; locomotory defects	ubiquitinated α syn deposits	actin cytoskeleton; apoptosis	(Ordonez, Lee and Feany, 2018)
	α syn WT, Δ 71-82, 1-120	neurons	no neurodegeneration for Δ 71-82, slightly increased for 1-120	Δ 71-82 no inclusions, more PK sensitive protein and less oligomers than WT; 1-120 more inclusions and increased PK resistance compared to WT		(Periquet <i>et al.</i> , 2007)
	α syn S129A, S129D	neurons	S129A suppresses dopaminergic neuronal loss; S129D enhances retinal degeneration	S129A more PK resistance, larger and more inclusions, more protein in sarkosyl-insoluble fraction; S129D similar to WT		(Chen and Feany, 2005)
	α syn WT, A30P, A53T, A56P and triple P mutant	neurons	triple P mutant reduces lifespan and motility most strongly; degeneration of dopaminergic neurons	triple P mutant is predicted to increase the formation of oligomers	A53T: mitochondrial proteostasis (TRAP1)	(Karpinar <i>et al.</i> , 2009)

* all A β 40/42 expressed with signal peptide

Table 5: List of rodent models of protein misfolding in Alzheimer's and Parkinson's diseases.

Misfolded protein	Name of the model	Construct expressed	Organismal/cellular phenotype	Protein aggregation behaviour	Pathways (genes) involved	Primary reference
<i>Alzheimer's disease</i>						
A β	<i>mouse</i>					
	PDAPP	APP V717F	dystrophic neurites and reactive astrocytes; synaptic loss and learning defects	ThS-positive extracellular plaques starting from 6-9 months		(Games <i>et al.</i> , 1995)
	Tg2576	APP K670N/M671L	dystrophic neurites and reactive astrocytes and microglia; impairment in Morris water maze	ThS-positive plaques at 11-13 months, some of which are Congo red birefringent; soluble species A β *56	immune response (progranulin); intracellular trafficking (Klc-1)	(Hsiao <i>et al.</i> , 1996)
	BRI-A β 40/42	BRI-A β 40/42	dystrophic neurites and reactive astrocytes around A β 42 plaques, but no behavioural abnormalities	ThS-positive plaques for A β 42; deposits in blood vessels; no aggregation for A β 40	immune response (progranulin); A β and lipid metabolism (ApoE)	(McGowan <i>et al.</i> , 2005)
	PS2APP	APP K670N/M671L + PSEN2 N141I	dystrophic neurites and reactive astrocytes and microglia; mild deficiencies in synaptic plasticity and in Morris water maze	congophilic A β plaques at 9 months		(Richards <i>et al.</i> , 2003)
	APP ^{SL} PS1KI	APP K670N/M671L/V717I + PSEN1 M233T/L235P	neuronal loss in hippocampus; impairment in working memory and motor tasks	ThS positive plaques and intracellular A β deposits		(Casas <i>et al.</i> , 2004)
	APPPS1	APP K670N/M671L + PSEN1 L166P	dystrophic neurites and reactive glia; cognitive impairment at 8 months	congophilic A β plaques starting from 6 weeks	immune response (progranulin); mitochondrial function	(Radde <i>et al.</i> , 2006)
	5xFAD	APP K670N/M671L/I716V/V717 + PSEN1 M146L/L286V	neuronal degeneration and loss; deficits in Y-maze test at 4-5 months	small and numerous ThS-positive plaques from 2 months; intracellular A β puncta from 1.5 months	immune response; cell differentiation; neurogenesis; histone modification; transcription; regulation of calcium binding; synaptic plasticity	(Oakley <i>et al.</i> , 2006)
	APP ^{NL-F} and APP ^{NL-G-F/NL-G-F}	APP K670N/M671L/I716V/(E693E) knock-in	neuroinflammation, synaptic alterations, memory impairment in Y-maze test	A β plaques first observed at 6 months for NL-F mice, and at 2 months for NL-G-F		(Saito <i>et al.</i> , 2014)
	<i>rat</i>					
tau	McGill-R-Thy1-APP	APP K670N/M671L/V717I	degenerating glutamatergic and cholinergic neurons surrounding plaques; learning and memory deficits at 3 months; limited neuronal loss at 18 months	ThS positive plaques; intracellular A β accumulation; soluble trimeric A β species	stress response; synaptic function; mitochondrial function; amino acid metabolism	(Leon <i>et al.</i> , 2010)
	TgF344-AD	APP K670N/M671L + PSEN1 Δ exon9	inflammation and phagocytosis of neuronal debris; significant neuronal loss; deficits in behavioural assays	widespread ThS positive plaques at 16 months; intracellular A β 42; soluble oligomeric A β species; Gallyas silver tau aggregates		(R. M. Cohen <i>et al.</i> , 2013)
tau	<i>mouse</i>					
	htau	genomic human tau in mouse tau knock-out background	memory impairment; significant neuronal loss between 8 and 18 months	phosphorylated and misfolded tau; fibrillar tau in somatodendritic compartments resembling pre-tangle state; PHFs in sarkosyl-insoluble fraction		(Andorfer <i>et al.</i> , 2003)

	Tau4RTg2652	tau 1N4R WT	abnormal axon morphologies and dystrophic neurites; slight motor impairment; memory deficit in Barnes maze	phosphorylated and misfolded tau; low levels of oligomers; no tangles		(Wheeler <i>et al.</i> , 2015)
	JNPL3	tau 0N4R P301L	motor and behavioural deficits	tau tangles in brain and spinal cord that are phosphorylated and ThS, Congo red and Gallyas silver positive; fibrils in EM; sarkosyl-insoluble material in aged mice	anti-apoptotic factors; microtubule-dependent intracellular trafficking; immune response	(Lewis <i>et al.</i> , 2000)
	pR5	tau 2N4R P301L	reactive astrocytes; apoptotic neurons	ThS and Gallyas silver positive tangles; granular accumulations of phosphorylated and misfolded tau; short fibrils in sarkosyl-insoluble fraction	advanced glycated end products (glyoxalase I); mitochondrial dysfunction and ROS; synaptic function	(J. Götz <i>et al.</i> , 2001)
	rTg4510	tau 0N4R P301L	atrophy in cortex and hippocampus; impairments in Morris water maze test; locomotory deficits	misfolded and hyperphosphorylated tau; tangles; sarkosyl insoluble material; fibrils seen by EM	insolubility of kinases, enzymes, chaperones	(Ramsden <i>et al.</i> , 2005)
	PS19	tau 1N4R P301S	activated microglia and astrocytes; neuronal inflammation; neurodegeneration	phosphorylated and insoluble tau; ThS, Congo Red and Gallyas silver tangles, globular and thread-like structures	cytoskeleton (GTPase-regulatory proteins)	(Yoshiyama <i>et al.</i> , 2007)
	TauΔK280	tau 2N4R ΔK280	synaptic loss	misfolded and phosphorylated tau; partially insoluble tau; tangles at old age		(Eckermann <i>et al.</i> , 2007)
	TauRDΔK280	tau 0N4R ΔK280	astrogliosis; loss of synapses and neurons	hyperphosphorylated and insoluble tau; Gallyas silver positive tangles; fibrils in sarkosyl-insoluble fraction		(Mocanu <i>et al.</i> , 2008)
	htau40AT	tau 2N4R A152T	reactive astrocytes and microglia; loss of synapses and neurons; neuronal hyperexcitability; memory deficits	hyperphosphorylated, misfolded tau; Gallyas silver and ThS positive tangles; sarkosyl-insoluble tau	autophagy and lysosomal pathways	(Decker <i>et al.</i> , 2016; Sydow <i>et al.</i> , 2016)
	hTau-A152T	tau 1N4R A152T	reactive astrocytes; behavioural deficits; neuronal hyperexcitability; age-related neuronal loss	misfolded, soluble tau		(Maeda <i>et al.</i> , 2016)
	<i>rat</i>					
	3R tau151-391	tau 3R residues 151-391	shortened lifespan; no neuronal loss	phosphorylated, misfolded and sarkosyl-insoluble tau; ThS and Congo Red positive tangles		(Filipcik <i>et al.</i> , 2012)
amyloid-β + tau	<i>mouse</i>					
	TAPP	APP K670N/M671L + tau 0N4R P301L	inflammation and dystrophic neurites surrounding Aβ plaques as in Tg2576; motor symptoms as in JNPL3	increased tau tangles compared to JNPL3; no change in Aβ deposition		(Lewis <i>et al.</i> , 2001)
	3xTg-AD	APP K670N/M671L + tau 0N4R P301L + PSEN1 M146V	synaptic dysfunction	intracellular Aβ followed by plaques; misfolded and hyperphosphorylated tau; ThS and Gallyas silver positive tau tangles	oxidative phosphorylation; ion transport; apoptosis; mitochondrial protein synthesis	(Oddo <i>et al.</i> , 2003)
	PDAPP/PS19	APP V717F + tau 1N4R P301S	stronger motor phenotype and reduced lifespan compared to PS19	spread of hyperphosphorylated tau in brain regions following Braak staging; accelerated formation of ThS positive tau tangles compared to PS19		(Hurtado <i>et al.</i> , 2010)
	5xFAD/PS19	APP K670N/M671L/I716V/V717I + PSEN1 M146L/L286V + tau 1N4R P301S	increased inflammation, loss of synapses and neuronal death compared to PS19 or 5xFAD	increased hyperphosphorylated and misfolded tau compared to PS19		(Saul <i>et al.</i> , 2013)
	TauPS2APP	APP K670N/M671L	mild cognitive impairment; no observable neuronal damage or loss	accumulation of SDS-stable Aβ species as in PS2APP; phosphorylated tau spreading with	oxidative phosphorylation	(Rhein <i>et al.</i> , 2009; Grueninger <i>et al.</i> , 2010)

		+ PSEN2 N141I + tau 2N4R P301L		age; Gallyas silver positive tau tangles; PHF-like fibrils in EM		
	PLB1 triple	APP K670N/M671L/ V717I + PSEN1 A246E + tau P301L/R406W	impaired synaptic plasticity; cognitive defects; altered glucose metabolism; sleep disturbances	ThS and Congo Red positive A β plaques; phosphorylated tau starting at the same age of 6 months		(Platt <i>et al.</i> , 2011)
	APP ^{swe} /tau ^{VLW}	APP K670N/M671L + tau 2N4R G272V/P301L/ R406W	more neuronal loss than single transgenic lines	increased tau phosphorylation and accumulation in sarkosyl-insoluble fraction compared to tau ^{VLW} ; increased plaque load compared to Tg2576		(Pérez <i>et al.</i> , 2005; Ribé <i>et al.</i> , 2005)
	tau/PS1/APP	APP K670N/M671L/ V717I + PSEN1 M146L + tau 0N3R WT	dystrophic neurites; inflammation	phosphorylated tau surrounding A β plaques but no tangles		(Boutajangout <i>et al.</i> , 2004)
	APP/PS1/rTg 21221	APP K670N/M671L + PSEN1 Δ exon9 + tau 0N4R WT	more dystrophic neurites compared to APP/PS1 but no increased loss of synapses or neurons	increased area of ThS positive amyloid deposits; misfolded and phosphorylated tau but no tangles		(Jackson <i>et al.</i> , 2016)
	APPOSK- Tg/tau264	APP Δ 693 + tau 3R/4R WT	accelerated synaptic loss, memory impairment and neuronal loss compared to single transgenic lines	intracellular and oligomeric A β ; phosphorylated tau; Gallyas positive tau tangles; fibrillar tau in EM		(Umeda <i>et al.</i> , 2014)
	App ^{NL-G- JF} /MAPT dKI	APP K670N/M671L/ I716V/(E693E) + MAPT knock- in	no effect on inflammation or memory compared to App ^{NL-G-F} model	accelerated phosphorylation of tau compared to MAPT knock-in only, but no tangles		(Saito <i>et al.</i> , 2019)
	APP/PS1 x rTauEC	APP K670N/M671L + PSEN1 Δ exon9 + tau 0N4R P301L	increased neurodegeneration and neuronal loss	increased spread of misfolded tau from the entorhinal cortex to distal brain regions; more amyloid plaques than in APP/PS1		(Pooler <i>et al.</i> , 2015)
<i>Parkinson's disease</i>						
α -synuclein (<i>asyn</i>)	<i>mouse</i>					
	Line D	<i>asyn</i> WT	loss of dopaminergic neuron terminals in striatum; motor defect	nuclear, ER-associated and cytoplasmic granular inclusions		(Masliah <i>et al.</i> , 2000)
	Line 61	<i>asyn</i> WT	loss of dopamine; motor symptoms; olfactory deficits	PK-resistant inclusions	membrane signalling (GnaI, Pde10a, Itpr1); extracellular matrix remodelling and inflammation (Hspg2, Lama5, and Lamb2)	(Rockenstein <i>et al.</i> , 2002)
	M83	<i>asyn</i> A53T	motor defects; no loss of dopaminergic neurons	fibrillar and ThS-positive protein		(Giasson <i>et al.</i> , 2002)
	hm ² α -SYN	<i>asyn</i> A30P + A53T	locomotory defects; loss of dopaminergic neurons	no cytoplasmic inclusions identified	mitochondrial function and oxidative stress; ubiquitin- proteasome system; ER stress	(Richfield <i>et al.</i> , 2002)
	Syn130m	<i>asyn</i> WT 1-130	specific loss of dopaminergic neurons in <i>substantia nigra</i> , presumably during development	no LB-like structures identified by different staining methods or western blot		(Wakamatsu <i>et al.</i> , 2008)
	<i>SNCA</i> -OVX	genomic <i>asyn</i> WT	locomotory defects; loss of dopaminergic neurons; reduced dopamine release; increased clustering of vesicles at dopaminergic synapses	high-molecular weight species but no PK resistant protein		(Janezic <i>et al.</i> , 2013)

	<i>A53T</i> transgenic	α syn A53T	locomotory defects; loss of dopaminergic neurons; reduced dopamine release; fragmentation of Golgi apparatus	inclusions found at axons and in vacuolar structures in dopaminergic neurons	lysosomal and autophagic pathways	(Lin <i>et al.</i> , 2012)
	3K	genomic α syn E35K + E46K + E61K	progressive locomotory defects; dopaminergic neurodegeneration; partially restored with L-DOPA	granular inclusions of PK-resistant α syn with S129 phosphorylation and C-terminal truncation; co-localisation with vesicles at synaptic terminals; lipid-rich accumulation		(Nuber <i>et al.</i> , 2018)
	<i>rat</i>					
	BAC transgenic	genomic α syn WT	changes in novelty-seeking and avoidance behaviour; reduced sense of smell; locomotory defects; dopaminergic neurodegeneration	accumulation of insoluble and PK-resistant protein; C-terminally truncated α syn in insoluble fraction		(Nuber <i>et al.</i> , 2013)